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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF A FOURTH 100 SPECIES, ALL PENICILLIA.

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THIS paper continues the investigation into the production of bacteriostatic substances by fungi of the "mould" type, which have been examined in sets of 100 (Wilkins and Harris, 1942, 1943*b* and 1944*b*). Results of the work described in the first two papers suggested that the Aspergilli and the Penicillia gave the most promising results; therefore, the third paper dealt with Aspergilli only and the present paper deals with Penicillia only. The importance of these two groups in the production of antibiotics receives continued confirmation. The fungi were grown in liquid culture media of different kinds and were tested by the method previously described (Wilkins and Harris, 1943*a*). The cultures were obtained from the Thom Collection in the United States *cia* the Lister Institute and the "Lister Number" is given in each case. The results are shown in the following list, where the symbol + indicates a positive, and the symbol — a negative reaction against the three bacterial types, *Bact. coli*, *Staph. aureus* and *Pseud. pyocyanea*.

LIST OF FUNGI EXAMINED.

Positive.

	Bact. coli.	*Staph. aureus.*	*Pseud. pyocyanea.*
P. avellaneum Thom & Turesson 984	+	+	—
P. avellaneum Thom & Turesson 3751	—	+	—
P. (*Scop.*) *brevicaulis* (*Sacc.*) Bain. 1362	+	+	—
P. (*Paecil.*) *Burci* (*Pollacci*) Thom 6164	—	+	—
P. canescens Sopp. 6607	+	+	—
P. carpentales 6608	+	+	—
P. (*Glio.*) *catenulatum* Gil. & Abbott 6597	—	+	—
P. chlorophaeum Biourge 6609	—	+	—
P. chrysitis Biourge 6610	—	+	—
P. chrysogenum Thom 5611	—	+	—
P. chrysogenum notatum ser. 3955	+	+	+
P. chrzaszczi Zal. 6611	—	+	—

	Bact. coli.	*Staph. aureus.*	*Pseud. pyocyanea.*
P. cinerascens Biourge 6521	+	+	−
P. claviforme Bain. 6613	+	+	+
P. cyano-fulvum Biourge 6615	−	+	−
P. (Glio.) deliquescens Sopp. 6598	−	+	−
P. expansum Link emmend. Thom 976	+	+	−
P. (Glio.) fimbriatum Gil. & Abbott 6599	−	+	−
P. fluorescens Sopp. 6621	−	+	−
P. gladioli Machacek 3994	+	+	−
P. godlewskii Zal. 3977	+	+	−
P. griseo-roseum Dierckx. 6623	−	+	−
P. implicatum Biourge 6524	+	+	−
P. italicum Wehm. 3499	−	+	−
P. janthogenum Biourge 6627	+	+	−
P. javanicum van Beyma 6628	−	+	−
P. lanoso-viride Thom 3972	−	+	−
P. lilacinum Thom 1719A	−	+	−
P. obscurum Biourge 6634	−	+	−
P. pfefferianum-spinulosum ser. 3942	+	+	−
P. roseo-maculatum Biourge 6516	+	+	−
P. Sartoryi Thom 6530	−	+	−
P. (Scop.) sp. 3867	+	+	−
P. tardum Thom 4046	−	+	−
P. tardum Thom 4047	+	+	−
P. terrestre Jensen 2515.	+	+	−
P. turbatum Westl. 6523	−	+	−
P. urticae Bain. 6650	+	+	+
P. vinaceum Gilman & Abbott 6518	+	+	−

Negative.

P. atramentosum Thom 6600 ; *P. aurantio-brunneum* Dierckx. 6525 ; *P. bacillosporum* Swift 6601 ; *P. baiiolum* Biourge 6527 ; *P. biforme* Thom 6602 ; *P. biourgeianum* Zal. 3540 ; *P. brefeldianum* B. O. Dodge 6604 ; *P. (Scop.) brevicaulis (Sacc.)* Bain. 580, 4048 and 4049 ; var. *alba* Thom 581 ; *P. brevicompactum* Dierckx. 6605 ; *P. camemberti* Thom 3970 ; *P. canditanteum* Westl. 6606 ; *P. carmino-violaceum* Dierckx. 5312 and 5848 ; *P. caseicolum* Bain. 602, 3969 and 6307 ; *P. chermesinum* Biourge 6517 ; *P. chrysogenum-notatum ser.* 3954 ; *P. citreo-roseum* Dierckx. 6612 ; *P. (Cit.) coeruleus* Sopp. 6595 ; *P. corylophilum* Dierckx. 6614 ; *P. corymbiferum* Westl. 4040 ; *P. decumbens* Thom 6519 ; *P. digitatum* Sacc. 6311 ; *P. Duclauxii* Delacr. 4043 ; *P. fellutanum* Biourge 6520 ; *P. flavi-dorsum* Biourge 6528 and 6620 ; *P. fusco-glaucum* Biourge 3975 ; *P. (Cit.) fuscus* Sopp. 6596 ; *P. glabrum ser.* 3945 and 3946 ; *P. (Paecil.) hibernicus* Kenn & Grimes 3532 ; *P. humuli* van Beyma 5810 ; *P. italicum* Wehm. 5615 ; *P. lilacinum* Thom 5618 ; *P. lividum* Westl. 6522 ; *P. luteum* Zuk. 1828 and 4045 ; *P. matris-meae* Zal. 6632 ; *P. Oledskii* Zal. 6526 ; *P. (Glio.) penicillioides* Cda. 1454 ; *P. pfefferianum-spinulosum ser.* 3937, 3938, 3939 and 3941 ; *P. roqueforti* Thom 5623 ; *P. roseo-pupureum* Dierckx. 6642 ; *P. (Glio.) roseum* Bain. 1290 ; *P. rugulosum* Thom 5624 ; *P. (Paecil.) sp.* 1241 ; *P. (Scop.) sp.* 3171 ; *P. terrestre* Jensen 3980, 3982 and 3983 ; *P. (Paecil.) varioti* Bain. 2030 ; *P. viridicatum* Westl. 3999 ; *P. Waksmani* Zal. 6529.

The abbreviations in the above list are explained as follows : " Cit." = Citromyces

of Wehmer; "Glio." = Gliocladium" of Corda; "Paecil." = Paecilomyces, and "Scop." = Scopulariopsis, both of Bainer. In view of controversy as to the validity of these genera we have included them all under the genus Penicillium. (P).

The fungus named as *P. carpentales* (no. 6 in the list), which was received from Dr. Kenneth B. Raper of the United States, is difficult to identify. The name "Carpentales" was proposed by Langeron as a generic name for Brefeld's *P. glaucum* and, later, Shear used it in the same sense and included certain other species. As a specific name "Carpentales" is, as far as we know, without definite authority.

The results in the above list show characteristic similarity to those in previous lists of Penicillia which have been tested. Those species which are especially promising are being investigated chemically and therapeutically at the Oxford School of Pathology.

In addition to the above mentioned "mould" fungi we have tested some 1500 individual fungi belonging to the "Larger Basidiomycetes" (Wilkins and Harris, 1944*c*), both by the above method and by a method specially devised for this type of fungus (Wilkins and Harris, 1944*a*). The total results of all species of fungi tested to date are given in the following table. The term "strongly positive" is applied to those metabolism solutions which produce a zone of inhibition greater than 4·5 mm., and the term "weakly positive" to those which produce a zone wider than 2 mm. The percentages are approximate.

Results of all Species Tested to Date.

	Total examined.	Strongly positive.	Weakly positive.	Negative.
Phycomycetes	30	—	—	100%
Ascomycetes	20	—	—	100%
Aspergilli	150	30%	20%	50%
Penicillia	200	20%	30%	50%
Basidiomycetes	730	10%	20%	70%

In percentage number of strongly positives the Aspergilli lead, with the Penicillia second and the Basidiomycetes third. The number of outstandingly good positives in each of these groups is relatively small and, from this point of view, there is less difference between them than the percentage figures lead one to suppose. Perhaps the most interesting point is that the antibiotic supremacy of the Aspergilli and the Penicillia is now seriously challenged by the Larger Basidiomycetes.

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MAMMARY TUMOUR INDUCING FACTOR AND GENETIC CONSTITUTION.

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IT has been reported in a previous paper (Dmochowski, 1944) that the mammary tumour inducing factor is present in normal organs of low cancer strain mice which were fostered by high cancer strain females. Bittner (1939) and others had previously shown that this factor can be obtained from tumour tissue, normal organs and blood of high cancer strain mice. The mammary tumour inducing factor retains its activity after drying (Bittner, 1941).

In testing for the presence of the mammary tumour inducing factor all previous workers used susceptible mice of the same strains as those from which the factor was obtained. These mice were either high cancer strain mice deprived of the mammary tumour inducing factor by being fostered by low cancer strain females or low cancer strain hybrid mice obtained by mating low cancer strain females to males of the high cancer strain from which the mammary tumour inducing factor was derived.

The present experiments were undertaken for the purpose of ascertaining whether the mammary tumour inducing factor can give rise to breast cancer in a susceptible strain of mice of a genetic constitution different from that of the strain from which it originated.

METHODS.

$C_{57} \times$ Strong A low cancer strain hybrid mice, born and raised in the Laboratories, were employed in the experiments as test mice, since previous observations indicated that these mice are susceptible to the development of breast cancer when inoculated with Strong A mammary tumour inducing factor. In females of this hybrid strain breast cancer has not been found to arise spontaneously. Likewise no case of mammary cancer has occurred in the strain of Little's C_{57} mice maintained in the Laboratories during the last four years. The incidence of breast cancer in Strong A breeding females originally descended from Bagg albino strain is, in our experience, about 96 per cent. at an average age of 12·6 months.

The mammary tumour inducing factor was obtained from two breast tumours which developed in S $\times$ R III low cancer strain hybrid mice. These tumours were induced by splenic grafts from "S" low cancer strain mice which had been fostered by high cancer strain females of the R III strain of Dobrowolskaja-Zawadskaja.

The tumour tissue was desiccated in a vacuum apparatus over phosphorus pentoxide. After two weeks' storage the tumour tissue was resuspended in distilled water in a dilution 1 : 4 and used for injections. Each mouse received 1 c.c. of this material subcutaneously.

The experiment was started two years ago (July, 1942) by mating C_{57} females to Strong A males. The $C_{57} \times$ Strong A hybrid mice when five weeks of age were separated according to sex, marked individually and divided as equally as possible into three groups.

Mice of the first group were injected with the resuspended dried tumour tissue. Males of this group were painted with oestrone twice weekly for a period of 12 months and females were forcibly bred by removing each litter within 24 hours after birth. Mice of the second group were not injected with tumour tissue. Males of this group were also painted with oestrone and females were forcibly bred. Mice of the third group were allowed to breed and to bring up litters in a normal way. Mice of the three groups were kept under similar conditions and on a diet of " Rat-cake " with an unlimited supply of tap-water.

RESULTS.

The results of the experiment are summarized in Table I.

TABLE I.

Strain of mice.	Experimental mice. Dried S × R III breast cancer hormonal factor.	Controls.
$C_{57} \times A$ females	63·6%	0%	0%
Forced breeding	7 out of 11 mice	13·4 months	0 out of 16 mice	22 months	0 out of 15 mice	22 months

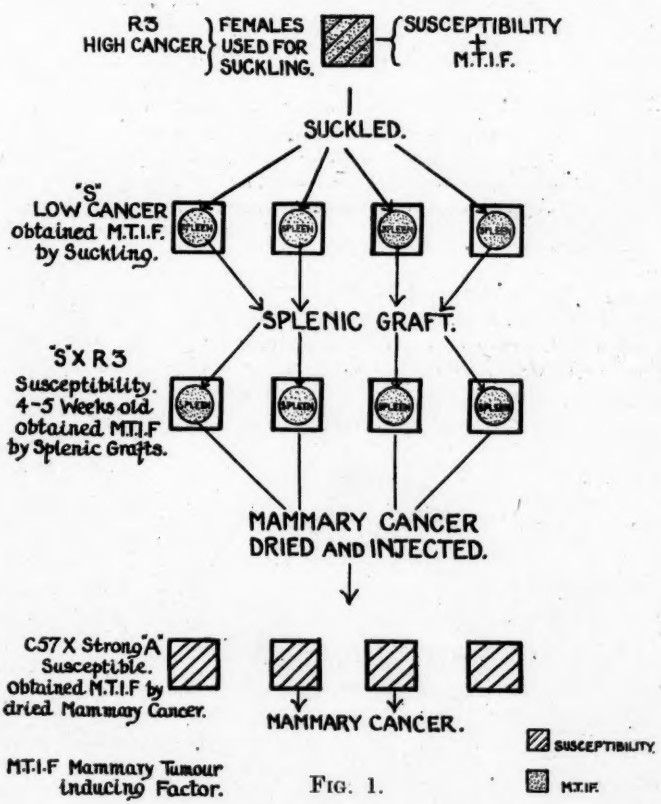
R3 HIGH CANCER
FEMALES USED FOR SUCKLING.
SUSCEPTIBILITY + M.T.I.F.
SUCKLED.
"S" LOW CANCER obtained M.T.I.F. by Suckling.
SPLEEN
SPLENIC GRAFT.
"S" X R3 Susceptibility. 4-5 Weeks old obtained M.T.I.F by Splenic Grafts.
MAMMARY CANCER DRIED AND INJECTED.
C57 X Strong "A" Susceptible. Obtained M.T.I.F by dried Mammary Cancer.
MAMMARY CANCER.
M.T.I.F Mammary Tumour inducing Factor.
SUSCEPTIBILITY
M.T.I.F.

FIG. 1.

It will be seen that no tumours developed in the litter mate control females which were forcibly bred and which were not injected with tumour tissue. This is in agreement with our previous findings that few or no tumours develop in susceptible mice after oestrogenic stimulation if the mammary tumour inducing factor is lacking (Dmochowski and Gye, 1944; Dmochowski, 1944).

The subcutaneous injection of R III mammary tumour inducing factor obtained from breast tumours which developed in S × R III mice induced breast cancer in C_{57} × Strong A females. No tumours developed in any of the C_{57} × Strong A males although they were painted with oestrone. The reason for this is obscure, and is being further investigated.

Thus breast cancer can be induced in C_{57} × Strong A hybrid mice females not only by the mammary tumour inducing factor derived from the Strong A high cancer parent strain, but also by a mammary tumour inducing factor from a totally different high cancer strain. The R III mammary tumour inducing factor can also be transmitted through mice of different genetic constitution ("S" and S × R III), and finally produce breast cancer in mice of a still different genetic constitution (C_{57} × Strong A). These apparently complex facts are represented in Fig. 1.

The results obtained in the present experiments show that the mammary tumour inducing factor present in dried breast cancer tissue of a susceptible hybrid strain induces breast cancer in susceptible hybrid mice which have an entirely different genetic constitution.

SUMMARY.

The results obtained in the present experiments show that the R III mammary tumour inducing factor can give rise to breast cancer in C_{57} × Strong A hybrid mice of a different genetic constitution from that of the strain from which the mammary tumour inducing factor originated. It can also induce breast cancer in this strain after transmission through two other strains of mice of different genetic constitution ("S" and S × R III).

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DMOCHOWSKI, L.—(1944) *Brit. J. exp. Path.*, **25**, 119.
Idem AND GYE, W. E.—(1944) *ibid.*, **25**, 115.

INFECTION OF MICE WITH *MYCOBACT. TUBERCULOSIS* (BOVIS) BY THE RESPIRATORY ROUTE.

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IT is generally considered that the mouse is highly resistant to *Mycobact. tuberculosis* of mammalian or avian origin.

The prevailing idea of the refractoriness of mice and rats to experimental tuberculosis is certainly borne out by the experiments undertaken by the Royal Commission on Tuberculosis (1907), in which it is stated that 5 mg. or more of a virulent culture given subcutaneously was necessary to produce a fatal disease in the mouse. It is noteworthy, however, that some authors—Stamatin (1939) and others—have described extensive lesions, more particularly in the lungs, following the intravenous injection of relatively small doses of bacilli; in addition, when introduced by other channels, e.g. by intraperitoneal injection, the organism shows a predilection for the lungs, although animals so inoculated do not necessarily succumb (Boquet and Nègre, 1921). Schwabacher and Wilson (1937) have shown that exposure of mice to a mist of virulent tubercle bacilli produces a well-marked, slowly progressive pulmonary disease which may prove fatal in from 10 to 21 weeks. An impression is thus gained that in the mouse the lungs are more vulnerable to attack than are other organs.

In the course of the experiments described in this paper, it was found that mice can be readily infected by bovine tubercle bacilli administered in the form of an aerosol of fine particles, and that pulmonary lesions were produced with relatively small doses. The effectiveness of this method was compared with the technique of intranasal instillation. So far as can be determined by macroscopic and histological examination, there was no difference as between the two methods in the distribution of the lesions and in the type of cellular reaction evoked.

METHODS.

Suitable apparatus for the exposure of mice to influenza virus particles in the form of aerosol mists has been described by previous workers—Wells and Lurie (1941), Edward, Elford and Laidlaw (1943), Loosli, Robertson and Puck (1943). Airborne infections with suspensions of tubercle bacilli have also been studied by Wells and Lurie (1941), Blaisdell and Hambleton (1942) in the rabbit, and by Schwabacher and Wilson (1937) in mice.

In the present investigation a special box was used for infecting mice. It is not proposed at this stage to give a detailed description of the apparatus, since the pilot model used was only suitable for the exposure of small groups of animals. A larger chamber embodying the same basic principles has since been constructed, and will be described in a subsequent communication. In brief, the chamber comprised a rectangular metal box divided into two compartments of equal size each measuring

18 cm. by 20 cm. by 20 cm. One section which received the atomized spray was used as a settling chamber for the sedimentation of coarse particles which might be introduced with the mist. The mice were placed in wire baskets in the other compartment through openings in the roof, closed during the experiments by plate glass covers sealed to the box with vaseline. One side of the settling chamber and the end wall of the exposure chamber were connected to a duct leading to the outside of the building. A portion of this duct was expanded to house an extraction fan to enable a current of air to be drawn through the box at the completion of each experiment. The movement of air from each chamber to the duct was controlled by sliding doors. The central partition dividing the box into two chambers contained openings also controlled by sliding doors, one of which was provided with a circular extension into the settling chamber housing a small fan, the operation of which ensured a uniform distribution of the mist in the exposure section (Fig. 1). The exposure chamber was provided with a safety valve to prevent the development of a positive pressure during exposure of the mice.

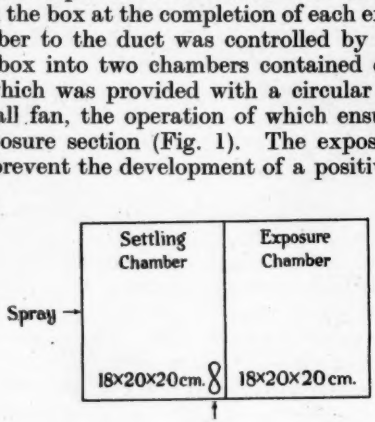

FIG. 1.

In order to ensure reproducible results, a bovine strain of full virulence for the rabbit has been used throughout the experiments. 20 ml. of a suitable suspension in normal saline solution was placed in a round-bottomed flask with a capacity of 500 ml. and was atomized under a positive pressure of 20 in. of Hg, using a modified form of the atomizer incorporated in the Collison inhaler, producing a particularly fine spray as described by Elford and Van den Ende (1942). The suspensions were prepared from weighed amounts of 5–7-day-old cultures grown on glycerol egg agar. The spray was operated for two minutes and the mist, after allowing the larger particles to settle for two minutes, was then transferred to the exposure chamber containing the mice. At the completion of each run, the chamber was disinfected by the introduction of steam containing formaldehyde vapour followed by a dilute ammonia vapour. After removing the mice from the box we took precautions to safeguard workers from possible infection with the highly virulent strains which were employed, by housing the animals in closed cubicles, thus preventing airborne particles from entering the room. Mice infected intranasally under ether anaesthesia were inoculated in the apparatus designed by Van den Ende (1943).

Comparison of Infection by the Intranasal Route with Exposure to an Aerosol Mist.

Several workers have attempted to ascertain the number of infective units of a virus, e.g. influenza virus, needed to infect mice (*a*) by exposure to an aerosol and (*b*) by intranasal instillation under anaesthesia.

Thus Edward, Elford and Laidlaw (1943) concluded that mice exposed for ten minutes to a tenfold dilution of a given aerosol of influenza virus developed lesions similar in extent to those induced by the intranasal instillation of 0·05 c.c. of fluid in

which the virus from 5 c.c. of undiluted aerosol had been concentrated; in addition it was noted that the infection contracted by exposure to aerosols was more regular than that following intranasal inoculation. Loosli, Robertson and Puck (1943) obtained results which substantially confirmed these findings: they concluded that passage of virus to the lungs by the inhalation method resulted in titres as high as those secured by intranasal instillation.

It is generally admitted that the intranasal method suffers from the defect that it is difficult to control accurately the dose which is given, while the amount of infective material reaching the lungs may vary considerably according to the depth of the anaesthesia of the mice.

In the experiments recorded in this paper, stock suspensions were prepared containing 20 mg. of *M. tuberculosis* in 20 ml. of normal saline solution. Tenfold dilutions were then made down to 1×10^{-7}. In the aerosol trials the mice were exposed to a series of mists prepared from suspensions ranging from 1×10^{-1} to 1×10^{-5} mg. per ml.; at the same time duplicate series of mice were inoculated intranasally under ether anaesthesia with 0·05 ml. of dilutions ranging from 1×10^{-2} to 1×10^{-7} mg. per ml.

In the case of mice exposed to the more concentrated preparations deaths from extensive tuberculous pneumonia occurred as early as the 18th day, and all the mice in a group were likely to succumb within 60 days. With progressively weaker concentrations, the interval between exposure and death was proportionately longer, with an increase in the number of survivors which showed tuberculous lesions. The smaller infective doses initiated pulmonary foci, which progressed to a certain stage and then tended to remain stationary or exhibited evidence of resolution. This result was not unexpected in a species which possesses some degree of natural resistance. On account of the propensity of the mouse to overcome a light infection, some of the animals were killed at a comparatively early stage to avoid overlooking minimal lesions which might have been obscured at a later date. In assessing the extent of the average tuberculous involvement in groups of mice, allowance had to be made for those animals which were killed in the preliminary stages. This point has to be appreciated in the interpretation of the tables.

Full details of a typical experiment are given in Table I. Groups each containing eight mice were exposed to aerosols prepared from saline dilutions of decreasing strength ranging from 1×10^{-1} to 1×10^{-5} mg. per ml., whilst similar groups were inoculated intranasally with suitable dilutions. The third column indicates the number of mice in a group (denominator) and the number with tuberculous lesions (numerator). The fourth column gives the number of days after infection before the animals died (†) or were killed (K). The extent of the lesions is indicated by plus signs. In subsequent tables these details have been condensed. A notation has been adopted as follows: Death of mouse from tuberculous pneumonia if more than half lung involved, 7; mouse killed with complete consolidation, 6; $\frac{3}{4}$ consolidation, 5; $\frac{1}{2}$ consolidation, 4; $\frac{1}{4}$ consolidation, 3; 5 to 20 scattered lesions, 2; less than 5 lesions, 1; no lesions, 0. The final column gives the total score expressed as a percentage of the maximum possible. It will be observed from Table I that there was little significant difference in the number of bacilli required to set up infection in the case of mice exposed to aerosol mists when compared with those infected intranasally. On the other hand, the aerosol suspensions tended to produce a more regular infection. Thus if the minimum certain infective dose of an aerosol is taken as 1×10^{-2} (12,000 bacilli), then all the mice exposed to these and to more concentrated mists exhibit recognizable lesions, whereas in the case of the intranasal route concentrations higher than the minimal infective dose do not always lead to 100 per cent. infection. The failure of a

TABLE I.—*Mice Exposed to Graded Doses of* Mycobact. tuberculosis: (*a*) *Aerosol*, (b) *Intranasal Instillation.*

	Weight of bacilli in mg./ml.	Estimated number of bacilli per mouse.	Proportion of mice affected.	Extent of lesions.								Percentage score* (lesions).
AEROSOL.	0·1	120,000	8/8	† 32 ++++	† 40 ++++	K 40 ++	K 42 +	K 69 ++++	K 83 ++++	† 102 ++++	K 150 +++	80
	0·01	12,000	8/8	K 42 +	K 42 +	K 42 + −	K 69 +++	K 83 +++	K 103 ++	K 150 +	K 150 + −	48
	0·001	1,200	7/8	K 42 − +	K 42 − +	K 42 − +	K 69 − +	K 83 + −	K 104 + −	K 150 +	K 150 0	19
	0·0001	120	3/8	† 15 0	K 42 0	K 42 0	K 69 0	K 83 + −	K 105 +	K 150 + −	K 150 0	12
	0·00001	12	0/8	K 69 0	K 83 0	K 105 0	K 105 0	K 126 0	K 126 0	K 150 0	K 150 0	0
INTRANASAL.	0·01	500,000	7/8	† 24 ++++	† 32 ++	† 38 +++	K 42 0	K 42 ++	† 65 ++++	† 68 ++	K 80 +++	68
	0·001	50,000	7/8	† 28 ++++	† 29 ++++	K 43 ++++	† 46 ++	† 53 ++	† 68 +++	K 80 +	K 150 0	68
	0·0001	5,000	7/8	† 26 ++	† 30 ++	† 30 +++	† 32 ++++	† 39 ++++	K 42 0	K 80 +++	K 150 +	66
	0·00001	500	7/8	K 43 ++	K 43 +	K 60 +++	K 60 ++	K 80 0	K 80 +	K 150 ++	K 150 ++	48
	0·000001	50	5/8	K 43 + −	K 43 + −	K 60 + −	K 60 0	K 80 + −	K 80 0	K 150 +	K 150 0	19
	0·0000001	5	0/8	† 55 0	K 60 0	K 60 0	K 80 0	K 80 0	K 80 0	K 150 0	K 150 0	0

* These figures must be regarded as approximate, since a proportion of the mice in each group were killed before the termination of the experiment.

++++ = complete consolidation.
+++ = 3/4 ,,
++ = 1/2 ,,
+ = 1/4 ,,

± = 5 to 20 lesions.
∓ = less than 5 lesions.
− = no lesions.

proportion of these mice to develop recognizable lesions is ascribed to difficulties inherent to the intranasal technique.

An experiment was made in order to confirm this supposition, and to ascertain whether the number of animals in groups subjected to mists of the same strength was likely to influence the extent of the pulmonary lesions. The latter consideration is of some importance, since the numbers of mice in a group exposed to the mists varied in different experiments from 3 to 12 animals. The capacity of the exposure chamber was about 7000 ml. From data furnished on p. 148 it will be seen that 12 mice exposed for 60 minutes would inhale about 36,000 ml., *i.e.* there would be about five respiratory turnovers per hour as against about one and a quarter turnovers for three mice. Batches of mice, containing 3, 6, 12 and 12 animals respectively, were therefore exposed to an aerosol mist containing 12,000 bacilli per c.c., whilst a second batch received an intranasal infective dose calculated to contain 50,000 bacilli. The results are shown in Table II. All the mice exposed to the mist developed tuberculous lesions and the majority died. The slight differences in the degree of consolidation which appeared in the various subgroups were probably without significance. On the other hand, 4 mice out of 24 exposed to intranasal infection were completely free from visible lesions.

Confirmatory evidence was thus obtained that for the production of a uniform infection the intranasal technique is not so accurate as exposure to a mist. In addition, and on the assumption that at each inhalation a proportion of the suspended bacilli is retained in the respiratory tract, it might be thought that the concentration of organisms in the air at any given moment would be directly related to the number of the mice, i.e. that the larger the unit the more rapid the removal of the bacilli. It would not have been surprising, therefore, if more extensive lesions occurred in the groups of the smaller numbers of animals. It is clear, however, that within the limits of the experiment, the number of animals inhaling tubercle bacilli in a restricted atmosphere has no material influence on the extent of the infection in each individual exposed to an aerosol of fixed strength for the same period of time.

TABLE II.—*Exposure of Groups of Varying Size to an Aerosol of Fixed Strength.*

	Number of mice in group.	Proportion affected.*	Percentage score (lesions).
Aerosol 12,000 bacilli	3	3/3	100
	6	6/6	95
	12	12/12	91
	12	12/12	94
I.N. 50,000 bacilli	24	20/24	79

* Survivors killed at 160th day.

Exposure to an Aerosol of Fixed Strength for Varying Periods.

In most of the experiments the period of exposure has been fixed arbitrarily at 60 minutes. Further trials have shown, however, that a uniform infection can be obtained when the period is shortened. Thus six mice were allowed to breathe an aerosol containing 15,000 bacilli per ml. After 10 minutes the mist remaining was swept out and the mice removed. The exposure was then repeated on further groups, the time being increased by 10 minutes at each stage. The results, as shown in Table III, indicate that maximum infection was reached after a period of 20 minutes.

TABLE III.—*Variations in Period of Exposure to Aerosol of Fixed Strength, (Aerosol 15,000 bacilli.)*

Period of exposure (mins.)	Proportion affected.*	Percentage score (lesions).
10	6/12	12
20	12/12	42
30	12/12	52
40	12/12	48
50	12/12	56
60	12/12	46

* Survivors killed at 90th day.

It would appear, therefore, that the concentration of bacilli remaining in the air after the lapse of 20 minutes is insufficient to affect the degree of pulmonary involvement. In other words, the bulk of the organisms seem to have settled out within 30 minutes. It is known that the loss in infectivity of a mist may be affected by the relative humidity of the atmosphere into which the droplets are introduced (Loosli, Lemon, Robertson and Appel (1943). Readings with a wet and dry thermometer taken from time to time showed that after the introduction of a non-infective mist into the chamber, relative humidity varied from 53 per cent. to 61 per cent. (average 57 per cent. $\pm$ 4). It is probable that these small differences affected the velocity of sedimentation to a negligible degree and can be ignored with safety.

Nature of Lesions.

The character of the tissue reaction induced in mice by tubercle bacilli inoculated intradermally or subcutaneously has been well described by Pagel (1940). He has noted that the most conspicuous feature is the appearance of " foam " cells, which he regards as large mononuclears filled with lipoid. These cells are nearly always surrounded by a thick hyaline capsule of collagenous fibres. Abscess formation, caseation and necrosis are seen only in exceptional instances, whilst no genuine tubercles or tubercle-like nodules are observed.

Generally speaking, the reaction of the lungs follows a similar pattern. There is little difference in character between the pulmonary lesions induced by exposure to an aerosol and those following intranasal instillation. The extent of the pulmonary involvement is governed to a considerable extent by the weight of infection. Massive doses tend to evoke a response which has a closer resemblance to the classical tubercle as seen in other species, but even in these instances although the bulk of the lung tissue is involved, the cellular reaction peculiar to the mouse still predominates. When the intranasal infecting dose is 12,000 bacilli or greater, some of the mice die between the 3rd and 15th weeks. The lungs are usually completely or nearly completely consolidated, the pulmonary tissue being replaced with greyish aggregates which are always firm and homogeneous. With smaller doses discrete lesions appear, varying from pinpoint foci just visible to the naked eye to larger lesions up to 3 to 4 mm. in diameter. In all cases irrespective of the extent of the lesions, the lungs are more voluminous than normal and only partially collapsed ; this is due to oedema. When exposed to minimal numbers of sprayed organisms the " tubercles " are usually fairly evenly distributed, but when the infecting dose is introduced intranasally the organisms tend to aggregate in the hilar region, although small subsidiary nodules may be found in other areas.

Histologically very little normal tissue can be seen in advanced cases. Aggregation of cells of a mononuclear type simulating formation of a tuberculous nodule

may be observed, but caseation and necrosis are almost completely absent, although karyokinesis of the cells in the central portion of these foci is a fairly constant feature. There is considerable proliferation of the alveolar epithelium, whilst the inter-alveolar tissue is distended by an oedematous infiltration. In some areas a peripheral zone can be traced comprising large cells with a vacuolated pale cytoplasm and a large, lobulated irregular nucleus ; these cells are obviously related to and may be identical with the foam cells described by Pagel. Large masses of acid-fast bacilli are distributed throughout the organ both intra- and extracellular in position.

Number of Viable M. tuberculosis *in Aerosol Mists Necessary to Produce Infection.*

The number of infective particles inhaled by a mouse is dependent on the length of time of exposure to an aerosol mist, the strength of the suspension and the respiratory turnover. Loosli, Robertson and Puck (1943) estimate that the respiratory minute volume of the normal mouse is about 50 ml. Data based on this assumption showed that not more than 10 per cent. of influenza virus particles breathed by a mouse reached the lungs. Edward *et al.* (1943) arrived at a similar conclusion. It is scarcely to be expected, however, that figures obtained from studies on influenza virus can be regarded as other than approximations, since in the present stage of our knowledge it is not possible to determine the exact number of viable, fully infective particles in a given suspension of virus. It is obvious that a dilution which is just large enough to produce visible pulmonary lesions may contain few or many aggregates. Experiments with the tubercle bacillus have the advantage that a much more accurate assessment of the number of units producing infection can be obtained than is the case with a virus suspension.

There seems to be general agreement that in the guinea-pig recognizable lesions can be evoked by suspensions containing not more than 50 virulent bacilli inoculated subcutaneously. In a comprehensive series of experiments by Lange (1926), the number of colonies produced by serial dilutions was compared with the infectivity of the same suspensions for the guinea-pig. It was estimated that from 1–5 organisms would cause recognizable infection. More recently Schwabacher and Wilson (1937), on the basis of direct counts, cultures and guinea-pig inoculations, concluded that the cultures used generally contained 50 per cent. or more of dead organisms, and that the minimal infective dose which could be relied upon to induce lesions was probably rather more than 10 bacilli.

In our experiments guinea-pigs were injected subcutaneously with graded doses of the bovine strains used for mice. It was found that a 50 per cent. lesion production rate (based on four guinea-pigs per dose) followed the inoculation of from 1/100,000,000 mg. to 1/1,000,000,000 mg. doses. According to direct counts, these dilutions contained from 5 to 50 organisms. All calculations in the tables have, therefore, been based on the assumption that the minimal infective dose for the guinea-pig is about 10 bacilli. It is possible that in accepting this figure we have erred on the high side, and that the number of organisms inhaled by the mice was slightly smaller than is indicated in the tables. In order to ascertain how many bacilli in an aerosol were needed to produce visible lesions in the mouse, it was necessary to estimate the number of particles present in the exposure chamber. To this end, a modification of the slit sampler elaborated by Bourdillon, Lidwell and Thomas (1941) was designed and constructed by Dr. E. Schuster. Briefly a metal platform carrying an ordinary microscope slide was allowed to pass horizontally beneath a $\frac{1}{2}$ in. slit. The period required for the complete traverse of the slide was one minute, in which time a $\frac{1}{2}$ cub. ft. of air had been withdrawn from the chamber. This air impinged on a slide coated with a thin layer

of a mixture of egg albumin 49 parts, distilled water 49 parts, and glycerol 2 parts. After collection of the bacilli, the slide was washed in 10 ml. saline solution and the suspension thus obtained was inoculated into guinea-pigs.

In a typical experiment, $\frac{1}{2}$ cu. ft. of the mist produced from a suspension containing 0·1 mg./ml. of *M. tuberculosis* was collected from the slide and the trapped bacilli suspended in 10 ml. of saline solution ; tenfold dilutions were then made for the inoculation of guinea-pigs (1 ml. amounts), two animals being used for each dilution. It was found that the guinea-pigs receiving the 1/1000 dilution containing the organisms from 0·1425 ml. of air developed lesions, whereas those receiving a 1/10,000 dilution were unaffected. It follows that each millilitre of air in the chamber contained $\frac{10}{0{\cdot}1425}$ bacilli. Experiments with slit samplers in tandem (new model coupled to a machine of the original design) gave a recovery efficiency of about 97 per cent., i.e. the greater proportion of bacilli were trapped in the sticky coating. If the respiratory turnover per minute of the mouse is accepted as 30 ml., then mice exposed for one hour to an aerosol mist prepared from a 0·1 mg./ml. suspension would breathe $\frac{60 \times 30 \times 10}{0{\cdot}1425}$ or approximately 120,000 bacilli. Table I shows that a mist prepared from a suspension of a strength of 0·0001 mg./ml. produced recognizable lesions in about a half the mice exposed, whereas a mist 10 times weaker was ineffective. The M.I.D. would thus appear to be in the region of 100 organisms ; the M.L.D., on the other hand, is probably greater than 120,000.

DISCUSSION.

By means of the apparatus described, mice were infected by exposure to airborne suspensions of *M. tuberculosis* introduced into a closed system. It would seem that the relatively high resistance possessed by mice to subcutaneous inoculation does not hold with the respiratory tract, since comparatively small numbers of bacilli were capable of setting up infection, although the disease produced was not necessarily progressive. The organisms localized in the inter-alveolar tissues, and evoked a reaction which bore a strong resemblance to that described by Pagel in mice injected subcutaneously or intradermally with large doses. Bacilli multiply exceedingly freely and may spread throughout the pulmonary system. The tissue reaction is, however, mainly proliferative with the appearance of numerous " foam " and mononuclear cells. Caseation and necrosis are not seen.

Schwabacher and Wilson (1937) found that the exposure of mice to 200,000 organisms for 3 minutes failed to induce visible lesions ; with stronger suspensions (20×10^6 and 300×10^6) macroscopic foci developed. Since the mists to which the mice were exposed were produced by an ordinary nasal atomizer, they undoubtedly contained numerous large particles which condensed on the wall of the flask used for exposure. The number of bacilli actually inhaled may, therefore, have been considerably smaller than is indicated by their figures.

Wells and Lurie (1941), by methods similar to those described in this paper, have furnished some interesting figures as to the number of inhaled bacilli which are necessary to evoke pulmonary lesions in the rabbit. The respiratory turnover was calculated at about 500 ml./minute. The number of bacilli carried into the lungs was found by exposing rabbits to airborne tubercle bacilli and making cultures from the lungs after 24 hours, the results being compared with the pulmonary lesions induced by comparable doses in other animals. Extensive to moderate tuberculous lesions were produced with 2000 to 3000 bacilli, whilst slight but recognizable foci followed inhalation of 20–30 droplet nuclei.

The figures which we have obtained for the mouse suggest that this species is almost as susceptible as the rabbit to infection by the respiratory route. It would appear that discrete foci are formed by relatively small doses administered in the form of an aerosol mist or by intranasal instillation under anaesthesia. Since a more uniform infection follows the exposure technique, this method is to be preferred when reproducible results are desired.

SUMMARY.

An apparatus is described for the exposure of mice to known doses of airborne tubercle bacilli. A dose of about 100 organisms will infect some mice ; 1000 organisms will infect nearly all.

Recognizable tuberculous foci are produced in a proportion of mice following the intranasal instillation of about 50 organisms, but this technique is not so accurate as exposure to an infected mist.

Concentrated aerosol mists induce extensive pulmonary consolidation within 60 days; weaker suspensions are not lethal, but evoke discrete, slowly progressive foci.

The numbers of viable bacilli in aerosol mists used for infection of mice have been estimated by the inoculation of guinea-pigs.

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MAGNESIUM POTENTIATION OF ADENOSINE TRIPHOSPHATE SHOCK AND OTHER SHOCK-INDUCING MEASURES.

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EVIDENCE has been obtained by Bielschowsky and Green (1944) indicating that muscle adenosine triphosphate (ATP) exists in the form of the magnesium salt, and that this is a more potent shock-inducing agent than the Na or K salts. Dubois, Albaum and Potter (1943) obtained increased yields of ATP from muscle and brain from animals killed during Mg anaesthesia. These observations as well as those concerning the *in vitro* effect of Mg on muscle adenosine triphosphatase (myosin) which, according to Lyubimova and Pevsner (1941) is inhibited [though Bailey (1942) did not confirm this], led us to study the effect of Mg salts on the action of ATP in the whole animal. The effect was dramatic, for the animal went into a profound state of " shock " within a few minutes with a dose of ATP far below the effective dose when given alone. It was found also that magnesium had a similar effect in precipitating " shock " when other shock-inducing methods were used. That a common factor exists in shock induced by quite different methods is obviously of great interest, and has led to a detailed investigation. Here there is only space to give a very condensed account of the more important findings, and attempt an explanation of the mode of action of magnesium. A full account of the cardiovascular changes is being published elsewhere.

The clinical condition of Mg anaesthesia and profound shock as seen in animals are similar. The term " shock " in the present context only implies that the condition resembles that induced by lethal doses of ATP (Green, 1943). For descriptive convenience it is assumed, prior to the discussion, that the action of Mg is to potentiate ATP and other forms of shock and not *vice versa*. Unless otherwise stated the magnesium salts of ATP and related compounds were used.

Magnesium Potentiation of the Shock-inducing Effects of Adenosine Triphosphate and Its Derivatives.

This was observed in every species tested, viz. mouse, rat, guinea-pig, rabbit and cat. Many different combinations of doses and routes of injection have been tried. An illustrative example is as follows : The rat is given 75 mg. $MgSO_4$ 7 H_2O (7·5 mg. Mg./100 g. body wt.) subcutaneously followed by 15 mg./100 g. body wt. ATP intraperitoneally. Respirations almost immediately become rapid and shallow, but the rate rapidly and progressively diminishes and the movements become dyspnoeic and forceful. The muscles become flaccid, and usually within 1–5 minutes the animal is in an anaesthetic state, with an absent or feeble corneal reflex. The heart rate slows appreciably during the period of increased respirations and then quickly returns to

the normal level. The beat then becomes more forceful and soon a slower but progressive decrease in rate sets in. The body temperature begins to fall immediately after the injection of ATP and rapidly reaches very low levels. The animal remains in this profound state of prostration for a very variable period, extending up to 18 hours, before death ensues or gradual recovery sets in. An animal may remain in this state, which on casual inspection simulates death, and yet after many hours recovery sets in. These time-relationships vary of course according to the doses used. With approaching death the heart and respiratory rates are extremely low, whilst the temperature sinks to almost that of the room air.

The mouse is the most useful animal for routine tests, for after Mg treatment (7·5 mg. Mg/100 g. body wt. intraperitoneally) as little as 10 mg. of ATP/100 g. mouse, intramuscularly, will precipitate shock, though at this dosage, recovery ultimately occurs in a high proportion.

The effect on the cat should be noted, for its reaction differs in some ways from the other species. It vomits soon after the ATP injection, and the respirations for a few minutes become extremely rapid and shallow, with signs of severe air-hunger. The respirations then progressively slow, but they remain relatively rapid and shallow, with dyspnoea until death is approaching. The heart-rate also quickens, the beat is forceful, and this state persists until death is imminent. With doses of Mg and ATP of the same order as in other species, reflex activity is not entirely lost until near the end though there is profound prostration, the pupil is widely dilated and the cornea only feeble sensitive.

TABLE I.—*Fatal Doses* of Magnesium and Adenosine Triphosphate when Both Injected Concurrently Compared with that of either Injected Alone by Similar Route.*

	Combined (mg./100 g. body wt.)				Alone (mg./100 g. body wt.)	
Species.	Mg.	Route.	ATP (Mg salt).	Route.	Mg.	ATP (Mg salt).
Mouse	6	I.P.	25	I.M.	12	75
Rat	7·5	I.M.	20	I.P.	12	50
Rabbit	5	I.P.	10	I.M.	15	20
	2	I.V.	1	I.V.	15	2
Guinea-pig	7·5	S.C.	15	I.P.	20	25
Cat	7·5	I.M.	10	I.P.	20	150†

* The figures indicate the order of dosage. The very large groups of rats or mice of the same strain, weight and sex necessary for a statistical analysis have not been available, since the broad nature of these investigations makes many demands on stocks obtained from various sources. In other species only a few uncomplicated tests of lethal dosage have yet been made.

† Based on previous findings with muscle fractions.

There is a reciprocal relationship between the amounts necessary so that, for instance, in the mouse 0·75 mg. Mg and 2·5 mg. ATP produce the same effect as 0·25 mg. Mg and 7·5 mg. ATP. The almost immediate effect of injecting both ATP and Mg by separate routes (*cf.* 6·0 mg. Mg i.p. + 25 mg. ATP i.m./100 g. mouse) can be reproduced by Mg given alone in doses of 10–15 mg. The important difference is, however, that whilst Mg anaesthesia usually rapidly disappears unless further doses of Mg are given, Mg + ATP anaesthesia persists for many hours. It is in fact impossible to reproduce the whole syndrome with a single dose of either Mg or ATP alone, for even with an enormous dose (except intravenous) of ATP (e.g. 200–300 mg. ATP i.p./100 g. mouse) the onset of the anaesthetic state is never quite so rapid as with a small dose (e.g. 10 mg.) in the Mg-treated animal. Such a high dosage of ATP is moreover quickly lethal, death occurring shortly after the anaesthetic state is reached. With a small but ultimately lethal dose (50 mg. ATP i.p.) the anaesthetic stage does not appear until 1–2 hours or even much longer after injection. The most important feature of Mg potentiation is the dramatic onset of the stage where all bodily activities

are strikingly reduced. It is this feature which has been used as an index of Mg potentiation when other shock-inducing methods were used.

The lethal dose of ATP is, as would be expected, much reduced by previous Mg treatment; about half the anaesthetic dose of Mg reduces the lethal dose of ATP by about two-thirds. It must be stressed that such figures as are given (see Table I) represent the general order of dosage. In all these experiments there is a wide dispersal about the mean; the individual tolerance even in litter mates varies very widely.

It was obviously important to find to what degree this Mg potentiation was specific for ATP, particularly in regard to its breakdown products. It was found that the removal of successive phosphoric acid groups yielded compounds all of which were potentiated. Adenosine diphosphate, adenylic acid and adenosine all acted like ATP, though Mg potentiation showed a definite reduction with each stage of dephosphorylation. Deaminated compounds (e.g. inosine triphosphate) were also potentiated. A very significant finding was, however, that Mg did not influence the action of inorganic pyrophosphate nor of adenine. Since inorganic pyrophosphate after injection produces very similar effects to organic pyrophosphate, and is in fact much more potent than many of the purine compounds which are potentiated by Mg, the effect is not likely to be due to a simple summation of toxic effects. In general it would seem that Mg probably potentiates all the possible breakdown products of ATP provided the purine-pentose linkage remains intact. The degree of potentiation, however, varies directly with the toxicity of the substance, being highest with the salts of ATP itself, and even with these the Mg salt is more strongly potentiated than the less toxic Na salt.

The specific nature of the Mg + ATP effect is indicated by the failure of Mg to potentiate the toxic action of other tissue constituents, such as potassium and histamine. Other evidence is provided by the failure of the cations, Li, Be, Sr and Ba, which have chemical and biological affinities with Mg and Ca, to potentiate the action of ATP.

As calcium is the pharmacological antagonist to Mg its effects were studied in more detail. When injected at an early stage of Mg + ATP anaesthesia it brought about recovery which was, however, short-lived, the animal gradually returning to its former state. The second phase was not reversible by Ca, nor could animals be recovered by Ca when they had been in the anaesthetic state for from half to one hour. In fact the ultimate effect of Ca was to reduce the survival time, and further experiments showed that Ca, of all the cations tested, was the only one, other than Mg, which definitely increased the toxic effects of ATP. Amounts of 5–10 mg. Ca/100 g. body wt. reduced the lethal dose of ATP by about one-third. Its action differed from Mg, however, in that it was gradual rather than sudden, and was therefore similar to the effect of giving a higher dose of ATP by itself. Thus the action of Ca presents the apparent paradox that, whilst it temporarily antagonizes the action of Mg, given separately its action is in some degree similar. The facts that the acute onset of symptoms is only produced by Mg, and that the effects of Ca and Mg on the action of ATP on the heart are completely opposite, suggest that the mechanisms of their actions on ATP shock are quite different.

Potentiation of Other Shock-inducing Agents by Magnesium.

The immediate importance of the above findings was that they appeared to present a method for detecting the presence of increased amounts of, or perhaps the presence of, extracellular ATP or its metabolites in the body. If such a state were present following tissue injuries of various types, then Mg treatment should accelerate the

onset and increase the severity of the resulting shock. Incidentally it should significantly reduce the degree of tissue trauma necessary to a fatal result. This proved to be so in greater or less degree with every shock-inducing method tried. A few of the many methods used are detailed in Table II.

TABLE II.—*Examples of Various Procedures which, when Followed by the Injection of Magnesium* (50–100 mg./100 g. Body Wt.), have Resulted in the Rapid Onset of Complete Prostration in a Large Proportion of Animals.*

Shock-inducing agent.	Species.	Method used.†
Adenosine triphosphate	Mouse	ATP (Mg salt)—10 mg./100 g. body wt. I.M.
" "	Rat	ATP (Mg salt)—10 mg./100 g. body wt. I.P. (Mg given I.M. or S.C.)
Limb ischaemia	Mouse and rat	Clamping of hind limbs sufficient to produce partial ischaemia.
Burn	"	Immersion of shaved hind limbs and hind quarters (technique of Rosenthal (1943) for 15 secs. in water at 70° C.)
Intestinal obstruction	Rat	Simple tie of small intestine 2 cm. below pylorus—Mg injected 18 hours later.
Dehydration	Mouse and rat	2·5ml./100 g. body wt. 40 per cent. glucose. I.P.
Haemorrhage	Rat	Rapid bleeding from femoral vein—2 ml./100 g. body wt.
Trauma	"	Strong mallet blows—15 to both hind limbs.
Cyanide	Mouse and rat	0·2 mg./100 g. body wt. NaCN. I.M.
Atropine	Mouse	1 mg./100 g. body wt. I.M.
Sodium nitrite	"	10 mg./100 g. body wt. I.M.

* The routes of injection varied but were usually intraperitoneal. Only a very small percentage of control animals were rendered anaesthetic by the doses of Mg used.

† During the period of acute tissue trauma the animals were anaesthetized with ether and Mg injected when they were fully recovered.

Hind limb ischaemia.

The effect of clamping one or both hind legs was first studied. When Mg was injected soon after release of the clamp(s) the onset of severe shock was much more rapid, as judged by rate of temperature fall, onset of haemoconcentration, fall in the respiratory rate, etc., and the survival time was greatly reduced. The lethal period of limb ischaemia was reduced by about half in the Mg-treated animal, e.g. in the rat from 4 hours, in both legs to 4 hours in one.

Occasionally an animal rapidly became completely anaesthetic and remained in that state until death. This, the most striking feature following ATP + Mg treatment, was most erratic in its appeance when Mg was injected after the obstruction to the limb circulation had been removed. If, however, Mg was injected shortly after applying a clamp to even one leg, about half the animals rapidly went into the anaesthetic state after the injection of sub-anaesthetic doses of Mg. Most of the affected animals died without regaining consciousness at times varying from a few minutes to 5 hours. Some recovered whilst the leg clamp was still in position, whilst others progressively deteriorated and died even when the clamp was removed shortly after the Mg injection. A second and smaller dose of Mg prostrated some previously unaffected animals, but about a fourth of the group were usually quite refractory to repeated doses of Mg provided, of course, they were such as failed to produce anaesthesia in the control animals. The explanation of the more striking effect obtained in the early stages of limb ischaemia is not clear. It may be that the clamps used do not produce complete ischaemia as they do not grip the whole circumference of the femur. Tissue metabolites may therefore be coming from the limb more freely in the early

period than later, when increasing local oedema around the clamp is compressing previously unobstructed vessels. This idea is supported by the finding that when the clamps were applied more loosely a larger proportion of animals showed a rapid response to Mg injection. In one group of 6 mice so treated, all were quickly prostrate and 3 died within a few minutes.

In the rabbit, ischaemia of the hind limb was produced in a different way—by occluding the aorta just above the bifurcation for 4½ hours. Intraperitoneal Mg in doses of 19 mg./kg. body wt. (quarter anaesthetic dose) was given at hourly intervals from the start. Of 12 rabbits, 5 died during the period of ischaemia, and only one survived for longer than 2½ hours after removal of the aortic clamp. Of 15 controls none died during the period of ischaemia, and 10 survived the subsequent 2½ hours. The condition of the Mg-treated animals was much more serious from the start than that of the controls; in the latter there was no clinical deterioration until after the aortic clamp was removed, whereas in the Mg-treated, indications of circulatory failure appeared in the first half hour of the experiment.

Though much more precise information is needed, it may be concluded that the effects of limb ischaemia are exaggerated in some way by the presence of Mg in the tissues. Calcium acted like Mg, except that just as with ATP, the fulminating action of Mg never appeared.

Burns.

Results were variable, but there were enough positive experiments to show definitely that the survival time was much reduced by Mg treatment after superficial burning. With a lethal burn, most or all of the animals became rapidly prostrate after Mg injection, but with a sub-lethal burn this immediate effect was rarely seen. The degree of burning required for a fatal issue was reduced in some cases by half, but consistent results in apparently uniform groups were not obtained. Some preliminary experiments indicate that smaller and deeper burns bring out the effect more consistently.

Trauma.

This shock-inducing method has not yet been extensively studied with a consistent technique. The effects of Mg treatment appeared to be similar to those following burns, one common feature being that rapid potentiation was only obtained following lethal amounts of tissue damage. Much indirect evidence was obtained in performing laparotomies (usually in control animals) in a variety of experiments. The injection of Mg after recovery from the anaesthetic frequently made the animal prostrate, in which state it sometimes remained until death. The main tissue trauma involved appeared to be that to the abdominal wall, though a less obvious cause of tissue damage may be a disturbance of the blood supply to the abdominal viscera.

Intestinal obstruction.

Only two groups of 6 rats were observed (see Table II), but these were sufficient to show that the injection of Mg at a time when the stomach was grossly distended with fluid rapidly made their condition worse. Six of the 12 died within a few minutes, and the remainder were soon prostrate and died much earlier than the controls.

The effect of Mg following obstruction of the blood supply to a segment (3 in.) of small intestine for 2 hours was inconclusive. It was noted in control rats that there was often no gross damage to the gut, nor obvious fluid loss, at the time when Mg was injected. This, when compared with the findings in simple intestinal obstruction

in the rat, suggested that extravascular fluid loss was an important factor in rendering the animal more susceptible to Mg.

Dehydration.

The last hypothesis was supported by the finding that the effects of the injection of hypertonic glucose solution were strongly potentiated by Mg. Soon after injecting about half the lethal dose of glucose solution, injection of Mg rendered every animal prostrate within a few minutes, and the survival times were much shorter than after a lethal dose of glucose alone. Saline solutions of equal hypertonicity acted likewise, except that the animals rapidly recovered. The difference between saline and glucose may be due to the much quicker attainment of equilibrium between blood and tissues after NaCl injection.

Haemorrhage.

Simple loss of water and electrolytes from the blood appeared to make the animal highly sensitive to Mg. If this sensitivity is associated with a fall in blood volume, then it should occur in the early stages after acute haemorrhage. It was not easy to demonstrate such a state after a slow but large bleeding or after repeated small bleedings, but after severing the femoral vein and allowing rapid bleeding (see Table II) the rat was, at any rate for a short period, highly sensitive to Mg injections. There was an interesting incidental finding. Control animals in which the femoral veins were dissected out but not cut responded to Mg injection to almost the same degree, though, unlike those bled, most of them recovered. It would seem likely that this increased sensitivity to Mg was the result of partial ischaemia of the limb following traumatic spasm of the femoral vessels.

In the rabbit no pronounced sensitivity was observed after repeated small bleedings, even up to a total volume of 10 ml. per kg. body wt. It may be noted here that there was no significant rise in the adenosine equivalent of the blood after a similar procedure (Stoner and Green, 1944*a*).

DISCUSSION.

The facts briefly described would seem to have an important bearing on the nature of shock since, however they are interpreted, they point to some common factor in the general effects of widely different forms of bodily insult. We are primarily interested in the hypothesis that this common factor is ATP or its derivatives. There seems to be, however, more than one plausible explanation for the experimental results. Some of the hypotheses which have been considered are:

Increased susceptibility of the nervous system to Mg as a result of anoxia.—Oligaemia, with a profound reduction in tissue oxidation, is known to result from all the shock-inducing methods used. If tissue anoxia is an important factor, then other methods of diminishing the available oxygen supply or the tissue respiration should also render the animal more susceptible to Mg. This proved to be so with the methods tried (see Table II). The injection of sodium cyanide, sodium nitrite or atropine increased the animal's sensitivity to Mg, the final picture being that of ATP "shock." The three substances produce a diminution in tissue oxidation by very different mechanisms, viz. inhibition of cytochrome oxidase, production of methaemoglobin, and in the case of atropine possibly by its effect on the respiratory centre. It seems clear then that general tissue anoxia is an important factor in decreasing the tolerance to Mg. Adenosine triphosphate might therefore, in relation to Mg, have the non-specific role of reducing tissue oxidation, for it is clear that after its injection basal metabolism is

much reduced. One difficulty in the way of this hypothesis is that the onset of acute effects appears very quickly after the Mg-treated animal has been traumatized. It does not seem likely, for instance (though obviously not impossible), that respiration in the central nervous system is significantly reduced immediately after rendering a limb ischaemic. Moreover, after an anaesthetic dose of Mg recovery is rapid (or more rarely the animal quickly dies), whereas after a sub-anaesthetic dose in the partially "shocked" animal, the anaesthetic state is very prolonged. Unless the disposal of Mg is less rapid than normal under these conditions, the recovery should be as rapid as in the animal made anaesthetic by Mg alone. The hypothesis in general cannot, however, be discarded, and must be weighed against the others.

Shock-inducing agents diminish the rate of excretion or disposal of injected magnesium.—Since all the shock-inducing methods used produce anuria or oliguria, the apparently enhanced action of Mg could be due to its diminished excretion. After double nephrectomy rats were little more susceptible to Mg injection than were control animals after a mock operation. Nor was the rat in which urine secretion had been suppressed by injecting pituitary anti-diuretic hormone any more sensitive to Mg injection. Mg estimations are very liable to technical errors, but we have made sufficient to indicate that the blood content of Mg in the supine rat after treatment with both Mg and ATP is no greater than in the fairly lively rat after treatment with the same amount of Mg alone, and is less than that in the anaesthetic state produced by Mg alone. A few estimations on whole rat brain also do not suggest that there is any significant rise in the Mg content in Mg-treated animals as the result of the injection of ATP. More observations are required on this last point, but the hypothesis in general has received no experimental support up-to-date.

Summation of the effects of some common factor in the mode of action of Mg, of ATP and of shock-inducing agents.—The clinical states of animals in deep Mg anaesthesia, in the later stages following ATP injection or after the release of hind-limb clamps appear similar. Mg itself, like ATP, produces a fall in body temperature, haemoconcentration, a moderate fall in blood pressure, peripheral vasodilatation and slow, forceful respirations. Similar changes occur in ischaemic shock in the rat, though it is not known whether the peripheral vessels are affected precisely in the same way. During Mg anaesthesia, however, these changes never become so profound as in shock, possibly because there is a relatively short interval between injection and recovery or death. There is thus a possibility of a summation of similar effects produced by different methods. The fact that there is no summation of the effects of Mg and of inorganic pyrophosphate (which has many effects in common with the organic polyphosphates) is the strongest point against this hypothesis. An alternative explanation of the similarities between Mg anaesthesia and ATP shock will be apparent in the later discussion.

Adenosine triphosphate or a derivative is the common factor potentiated by magnesium in shock-like states in general.—Before considering this hypothesis generally, let us consider some possible ways in which Mg might potentiate the action of ATP. Since it has been shown by Dubois, Albaum and Potter (1943) that an increased yield of ATP can be obtained from the tissues of the Mg-narcotized animal, the most probable explanation is that Mg inhibits the enzymic breakdown of ATP. The difficulty here is that Mg not only potentiates the tri (ATP), but the di and monophosphates of adenosine as well as adenosine itself. It also potentiates inosine triphosphate and probably a similar series of inosine compounds. It is unlikely that the various enzymes concerned in the breakdown of these compounds are all inhibited by Mg. It is not impossible, however, for there is evidence that under certain conditions Mg inhibits not only ATP-ase (*v.s.*), but also adenosine and muscle adenylic acid deaminases

(Ostern and Mann, 1933) and ATP deaminase (Stoner, Green and Bielschowsky, 1944). The idea can be rendered much more plausible by assuming that all these compounds are synthesized to ATP in the body without interference from Mg, whilst the immediate breakdown of ATP (by ATP-ase) is itself inhibited. There is some support for this assumption in the time-relations of the onset of symptoms when the various compounds are injected into the Mg-treated animal. Bearing on this point also is the finding that neither inorganic pyrophosphate nor adenine are potentiated by Mg, which is conceivably due to the inability of the tissues to make direct use of these substances in the synthesis of ATP.

Attempts have been made to demonstrate Mg inhibition of ATP breakdown by tissue *brei* and extracts *in vitro*. The Mg-treated mouse has been used as an extremely sensitive indicator of the amount of ATP breakdown which has occurred. The evidence so far, though not conclusive, favours the theory of enzymic inhibition by Mg.

That calcium also augments the toxic action of ATP (and also adenosine) must also be considered in this connection. The interpretation of this effect is complicated, since in the animal Ca is a pharmacological antagonist to Mg. Mg is, however, always dominant, a fact well brought out in observations on the opposed effects of Mg and Ca on the vaso-depressor action of ATP (Stoner, Green and Bielschowsky, 1944). It seems that the effect of Ca is more likely to be a summation of toxic effects, since it is seen with inorganic pyrophosphate, and it has not the specific features, particularly the rapid action seen with Mg potentiation. It is, of course, possible that Ca, though an activator of ATP-ase in physiological concentrations (Bailey, 1942; Needham, 1943), becomes inhibitory, though less so than Mg, at higher concentrations.

The evidence as a whole favours the view that Mg potentiation of these purine compounds is in some part due to inhibition of enzymic breakdown. Whether phosphatases alone, whether deaminases, or possibly a dependent relationship between the two processes are primarily concerned are questions not yet answered.

If such indeed be the full explanation of the mode of action of Mg, then the similarity previously noted between its effects and that of ATP in the intact animal could be ascribed to the same factor, i.e. the accumulation of ATP in pathological amounts in the tissues. Such an explanation of Mg anaesthesia was tentatively suggested by Dubois *et al.* (1943).

Another possible explanation of Mg potentiation that has to be considered is that Mg alters the permeability of some tissue cells to ATP and allied compounds, and thus favours a more rapid shift to, or a more rapid accumulation in susceptible organs. This possibility arose because of several findings which seemed difficult to interpret on the basis of enzymic inhibition. For instance, in the Mg-treated cat the intravenous depressor effect of ATP was greatly reduced—an effect which was first ascribed to a more rapid shift of ATP from the blood to the tissues through the more permeable capillaries resulting from Mg peripheral vaso-dilatation. It is now known that the effect is due to depression of the vagal centre by Mg, and since the vaso-depressor action of ATP in the cat (McDowall, 1944) is partly due to central vagal inhibition, the fall in blood pressure due to this is largely prevented by Mg.

Other relevant findings were as follows: When ATP is injected by routes other than the intravenous, it has no immediate effect on the blood pressure. In the Mg-treated animal such an injection occasionally produced a rapid fall in blood pressure. This again suggested that Mg, by increasing capillary permeability, favours a more rapid equilibrium between blood and tissues. Moreover it was found in the Mg-treated rat that the rise in the adenosine equivalent of the blood following ATP injection (I.M.) was more rapid than in controls. Both findings could be attributed to a less rapid deamination of ATP in the presence of increased tissue Mg, with a resulting

increased rate of absorption, except that it is difficult to reproduce them with ATP alone in much larger amounts. Barbour and Winter (1927, 1929) described the potentiation of certain antipyretics by Mg. This could be explained most easily by an increase in cell permeability. For these and other reasons (excluded for brevity) the possibility that some part of the action of Mg is due to changes in permeability cannot be excluded.

Whatever the exact mode of action of Mg, in the present connection it is simplest to postulate that it inhibits ATP breakdown, and thus leads to the accumulation of organic pyrophosphate with *sequelae* which simulate or *are* actually a state of shock. Assuming this is true, what evidence is there that the same mechanism is responsible for the potentiation of the effects of tissue trauma by Mg ?

If it could be shown that ATP or its breakdown products gain access to the blood from damaged tissues, under those conditions in which Mg potentiation is observed, then the latter thesis would receive weighty support. We have no direct evidence of this, but there is indirect evidence (mostly in the rabbit) that adenosine or adenosine compounds do increase in the blood after various forms of tissue trauma. A rise in the blood adenosine equivalent was observed in ischaemic shock and gravity " shock " (Stoner and Green, 1944*a*), as well as in glucose dehydration shock and traumatic shock (unpublished). In man a rise was found after a period of ischaemia to the legs (Stoner and Green, 1944*b*). We have failed to find any rise in the later stages of ischaemic shock in rats, though there are indications that such a rise may occur in the early stages of incomplete ischaemia of the limbs, i.e. at a time when the rat is particularly susceptible to Mg. There is thus some evidence that a secondary result of various shock-inducing methods is a disturbed balance of adenosine compounds in the tissues.

It has been noted, however, that various drugs, not producing the typical shock syndrome, are also potentiated by Mg, and it was suggested that the factor in common between the action of these drugs and that of shock-inducing agents might be the production of tissue anoxia. It was therefore interesting to find a rise in the adenosine equivalent of the blood in cyanide poisoning in the rabbit.

Two possibly important issues, one practical and one theoretical, arise out of this work. The first is that a sensitive method is provided for detecting abnormal amounts or a disturbed distribution of ATP and some of its metabolites in the whole animal. The second is its bearing on the nature of shock.

Some common state appears to be present after severe tissue damage, however produced. This state is probably one of general tissue anoxia, which may arise, in part at any rate, as a secondary result of oligaemia. Whatever is the process of events in the initiation of shock, it would seem that when a serious degree of general tissue anoxia has resulted, the release of toxic metabolites becomes a real possibility. As to the nature of these metabolites, ATP or related compounds, must on the evidence given be seriously considered. If, in fact, an accumulation of such compounds, for instance in the central nervous system, does occur, this can only happen when the enzymic breakdown processes are overwhelmed or the enzymes concerned are inhibited (e.g. by magnesium). It is conceivable that a state of rapid accumulation of organic pyrophosphate corresponds to the " irreversible " state of shock, and is an expression of severe functional damage to the central nervous system.

Such a theory does not exclude the possibility that the initial local and general effects of tissue damage are in some part due to the presence of extracellular ATP in the injured area. If, in fact, it is released in abnormal amounts, we have experimental evidence that local vaso-dilatation and fluid loss must result. In this way it could play a role, if only a small one, in the production of oligaemia, which, as previously

postulated, may lead to a general release of ATP from anoxic tissues. Moreover, unless it is all destroyed *in situ* (and there is evidence that this is not so), it must, on this hypothesis, be responsible in some degree for the early general effects of local tissue damage. In this connection the fact that Mg potentiates the effect of limb ischaemia at a very early stage should be recalled. In shock due to uncomplicated haemorrhage ATP could, of course, have no local role, but only come into the picture as a secondary result of severe oligaemia. Here again it may be significant that a profound degree of blood loss was necessary before Mg potentiation became evident.

Much more evidence would be required before any one of these hypotheses could be regarded as proved, but they may be useful at this stage as indicating a possible way in which the apparently opposed theories of the cause of shock, viz. local fluid loss, toxic metabolites and depression of the nervous sytem could be reconciled.

SUMMARY.

The injection of magnesium salts greatly increases the sensitivity of the intact animal to adenosine triphosphate and some of its derivatives, including adenosine. Magnesium inhibition of enzyme(s) concerned in the breakdown of adenosine triphosphate is the more likely explanation of the effect, though other possible mechanisms are considered.

The injection of magnesium salts increases in varying degree the response of animals to a wide range of shock-inducing methods—tissue ischaemia, burns, intestinal obstruction, dehydration, haemorrhage and trauma. The evidence suggests that in all these states one common factor responsible for this effect may be the general tissue anoxia resulting from oligaemia.

Some evidence is presented that under certain conditions adenosine compounds increase in the blood after various forms of tissue trauma, including uncomplicated extravascular fluid loss.

That a disturbed balance of adenosine triphosphate in the tissues is responsible for the increased sensitivity of the magnesium-treated animal to shock-inducing measures is considered to be a reasonable but unproved hypothesis.

The enhanced sensitivity of the magnesium-treated mouse to adenosine triphosphate and some related compounds may form the basis of a useful biological test for the detection of such compounds.

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MUSCULAR FATIGUE IN THE ALBINO RAT, AND ITS RELATIONSHIP TO THE THYROID.

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THE susceptibility of patients with thyrotoxicosis to muscular fatigue has been recorded by numerous authors—Eason (1927), Joll (1932), Crile (1932*a*), and Hertzler (1935). The muscular sluggishness of the myxoedematous patient is pathognomonic. Studies on spontaneous activity in the rat by Richter (1933) and Hall and Lindsay (1938), using the revolving cage technique, have shown that thyroidectomy and administration of thyroid substance produce an increased susceptibility to fatigue. Various authors have suggested that the effect in these cases is due to interplay between the pituitary-thyroid axis and the adrenal cortex. Salter (1940) concluded that there is a peripheral antagonism between the thyroid and the adrenal-cortical hormones. Marine (1930) stated that there was an adrenal hypofunction in thyrotoxicosis, while Crile (1932*b*), on the other hand, believed in an adrenal hyperfunction. Numerous authors—Gans and Miley (1927), Hartman, Brownell and Lockwood (1932), Heron, Hales and Ingle (1934), Ingle (1938, 1940*a*, 1940*b*)—have shown the effect of adrenalectomy on muscular work and the value of adrenal cortical extracts in offsetting fatigue. This paper describes studies of the effect on muscular fatigue in the rat of thyroid hypofunction produced by allyl-thiourea, rape seed diet (Kennedy and Purves, 1941), and thyroidectomy.

METHODS.

Healthy male rats of a Wistar strain were used, all being approximately of the same age (4 months) at the time of experiment. Column 1 of Table I shown below gives the method employed with each group.

The apparatus used was essentially the same as that employed by Heron, Hales and Ingle (1934). The only modification was the provision of a lever to record on a slowly-moving kymograph, thus enabling calculation of the rate of work at any desired time to be made. The anaesthetic employed was sodium barbital given intraperitoneally in a dose of 35 mg. per 100 g. of body-weight. During long-continued runs further 5 mg. doses were given in the same manner to maintain an even depth of anaesthesia. The tendon of the right gastrocnemius muscle was freed from the surrounding connective tissue, but the skin over the belly of the muscle was left intact in order to minimize shock and excessive drying of the surface. The weight lifted at each afterloaded contraction was 60 g. The stimulating apparatus, as with that of these workers, produced maximal stimuli three times per second. The stimulus was applied by insertion of pin electrodes directly into the muscle. Throughout the experiment care was taken to maintain the normal temperature and to give sufficient saline intraperitoneally to prevent dehydration. The results are expressed as the total work

in kilogram-centimetres per gramme of muscle. The weight of the latter was determined at post-mortem examination immediately after fatigue was complete. The post-mortem examination included a careful examination of the neck and abdomen for thyroid and adrenal changes.

RESULTS.

These are summarized in Table I.

TABLE I.

Group.	Mean body weight (g.).	Number animals.	Total work performed. Kg. cm. per g. muscle. (Mean.)	Standard deviation.	Mean time of performance (hours).	Remarks.
1. Controls	193	11	1907	414	20	Unfatigued.
2. Thyroidectomized 6 wks previously (*a*)	179	12	12·8	16·1	12 mins.	Fatigue complete.
2. Thyroidectomized 6 wks previously (*b*)	161	8	1434	299	20	Unfatigued.
3. Thyroidectomized 6 weeks previously. Injection 50 μg. thyroxine sodium per day, 3 days before experiment	210	3	1836	434	20	,,
4. Thyroidectomized 6 weeks previously. Injection 75 mg. D.O.C.A. 18 hours before experiment	147	7	1440	158	20	,,
5. As Group 1, but 10 mg. allylthiourea by mouth daily for 21 days before experiment	180	3	294	294	3	Fatigue complete.
6. As Group 1, but 64 mg. thyroideum siccum by mouth daily for 21 days before experiment	98	6	4·3	3·3	1 hr. 50 mins.	,,
7. Normal rats fed on rape seed diet for 3 months before experiment	242	6	138	6·7	2 hrs. 30 mins.	,,

While normal control rats could maintain their work at a high level for 20 hours, most of the thyroidectomized, those fed on a rape seed diet, and those dosed with allyl-thiourea were able to perform a relatively small amount of work, and were completely fatigued in a fraction of the time.

A proportion of the thyroidectomized rats (shown as Group 2*b*) showed a much higher rate of performance. Statistically, the difference between even this group (2*b*) and the control group is significant, as the possibility of these differences being due to chance is less than 1 in 20, calculated according to Fisher (1928). The completeness of the thyroidectomy must be open to doubt in these cases, and the likelihood of the development of an accessory thyroxine-producing mechanism cannot be overlooked.

Group 3 : The administration of 50 μg. of thyroxine sodium for three days prior to the experiment brings the work performance to the same level as the controls. Statistical analysis shows that the probability of this group being significantly the same as the control group is seven chances in ten, and recovery can therefore be assumed to be complete.

The administration of desoxycorticosterone acetate to thyroidectomized(Group 4) rats raises the total work performance to a level much higher than that of the completely thyroidectomized, but it is significantly lower than that of the controls.

The probability of this deviation from the control group being due to chance is less than 1 in 100.

Although the number given allyl-thiourea (Group 5) was small, their mean work performance is significantly low. Calculated by the same methods used above, the probability of the figures being chance deviations from the normal control group is less than 1 in 1000.

The result of administering thyroideum siccum by mouth for 21 days (Group 6) is a total work performance lower than in any of the experimental groups.

The size of the thyroids, as examined post mortem, was three to six times that of the control group in those rats fed on the rape seed diet and those rats dosed with allyl-thiourea.

The thyroids of the rats of Group 6 who had been given 64 mg. of thyroideum siccum per day for 14 days were the same as those on the control diet.

One result of interest obtained during the course of the experiments is not included in the above table. A group of three animals fed on rape seed diet for the same period as Group 3 were allowed to run to complete fatigue on the apparatus in two to three hours. At this stage 150 g. of thyroxine sodium was injected intraperitoneally. The stimulus to the muscle was not stopped, and after a short period of time, recovery was seen to occur in the height of the contractions. One such animal continued unfatigued up to 43 hours following the injection. This rapid action of injected thyroxine is in direct contrast to the delayed action of the hormone on metabolism. This observation of an immediate response to thyroxine requires confirmation.

DISCUSSION.

It will be seen from the above results that the effect of administering allyl-thiourea, of adding rape seed to the diet, and of thyroidectomy is to reduce greatly the ability of the rat muscle to perform work. This is apparently the result of a deficiency in thyroxine, as the administration of this substance leads to recovery. On the other hand, administration of excess thyroxine is inimical to efficient muscular contraction in the rat. This confirms the previous work of Richter (1935) and Hall and Lindsay (1938), who used for their experiments the revolving cage technique. In this work it was postulated by the latter that the "depressive effect of thyroidectomy could be alleviated by proper thyroid substitution, *avoiding overdosage*." The results from our experiments with the administration of thyroideum siccum confirm this in the artificially-stimulated rat muscle under more accurately controlled conditions than those of spontaneous activity. Apparently there must be an optimum concentration of thyroxine present for the production of maximum muscular work. Alteration upward or downward brings a decrease in the ability to perform work, and earlier fatigue.

That there are other factors involved is evident from the fact that in Group 4, Table I, a recovery in the work output of the thyroidectomized animal is obtained by the use of desoxycorticosterone acetate. This might suggest that adrenal deficiency plays some part in the fatigue of the thyroidectomized animal, but it is not inconsistent with the suggestion that under these conditions desoxycorticosterone acetate can to some extent take the place of thyroxine in muscle metabolism.

SUMMARY.

1. The gastrocnemius muscle preparation in the thyroidectomized rat, when stimulated directly, fatigues more readily than the control. Thyroxine counteracts this effect.

2. Animals with hyperplastic goitre, produced by goitrogenic diets or by allyl-

thiourea, show similar earlier muscle fatigue and lower output of work than control animals.

3. Oral administration of thyroid substance produces in the normal rat early fatigue and a lowered output of work.

We have to acknowledge our indebtedness to Prof. C. E. Hercus and the Goitre Research Staff for the original suggestion which prompted this work. Also to Dr. W. Griesbach for thyroidectomizing a number of animals, and to Mr. H. Manson for technical assistance.

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THE NUCLEIC ACID AND NUCLEOTIDE CONTENT OF TUMOURS.

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RECENT spectrophotometric work (Caspersson, 1936, 1940*a*, *b*, *c*, *d*, 1941 ; Caspersson and Schultz, 1939, 1940 ; Mitchell, 1942) has directed attention to the part played by the nucleic acids and their derivatives in relation to tissue growth, in rapidly growing embryonic (Caspersson and Thorell, 1941*a*, *b*) and neoplastic (Caspersson and Santesson, 1942) tissues. It has previously been shown (Davidson and Waymouth, 1943, 1944*a*, *d*) that ribonucleic acids are widespread in animal tissues, and are present in high concentration in the rapidly growing tissues of the sheep embryo. An attempt is made here to investigate their occurrence in tumour tissue.

Euler and Schmidt (1934) and Edlbacher and Jucker (1936) quote high figures for the total nucleic acid content of tumours, based on purine nitrogen estimations, while Berenblum, Chain and Heatley (1939) found that the nucleoprotein phosphorus (NPP) content of the Jensen rat sarcoma was higher than in most normal tissues. Some earlier results (Petry, 1899 ; Wolter, 1913 ; Enselme and Enselme, 1927 ; and Roffo and Pilone, 1930) indicated a high nucleic acid content for tumour tissues, though Wells (1912) and Wells and Long (1913) did not find high figures for the purine nitrogen of tumours.

The importance of making comparisons only between the tumour and its tissue of origin in studies of glycolysis mechanisms has been stressed by Berenblum, Chain and Heatley (1940), whose views have, however, been criticised by Burk (1942). Where the nucleic acid contents have been compared, the tumour is usually found to be richer in nucleic acid than the tissue of origin (Fujiwara, Nakahara and Kishi, 1937 ; Dickens and Weil-Malherbe, 1943 ; Rondoni, 1941). Kohman and Rusch (1941), using radioactive phosphorus, have observed an increased rate of nucleoprotein metabolism in neoplastic tissue.

Normal mammalian tissues from adult and embryo contain both desoxyribonucleic acid (thymonucleic acid) and ribonucleic acid (Davidson and Waymouth, 1944*a*, *d*). In the liver, and probably in other tissues as well, the ribonucleic acid is found in the cytoplasm (Davidson and Waymouth, 1944*c*), where it may occur in the form of phospholipin-ribonucleoprotein complexes. Desoxyribonucleic acid is solely a nuclear constituent.

According to Koller (1943*a*, *b*) those tumours with a high cell division rate have a high concentration of desoxyribonucleic acid in the nuclei, whereas in tumours with cells of low division rate, high cytoplasmic volume and large nucleoli, ribonucleic acid is abundant as a cytoplasmic and nucleolar constituent. This view has been criticised by Dounce (1943) on the grounds that the isolated nuclei of rat hepatoma cells are no richer in desoxyribonucleic acid than are normal liver-cell nuclei.

Neuberg (1905), Neuberg and Milchner (1904) and Beebe and Shaffer (1905) quote figures for the pentose content of tumours, but the methods then employed did not justify the assumption that the material estimated was derived from ribonucleic acid or ribonucleotides only. There is some evidence that ribonucleic acid is present in the cytoplasm of malignant cells (Caspersson, Nyström and Santesson, 1941 ; Caspersson and Santesson, 1942 ; Mitchell, 1942), and Claude (1940) has shown that tumour-producing cell-free sarcoma extracts contain pentose nucleic acid in the form of phospholipin-ribonucleoprotein complexes.

PROCEDURE AND METHODS.

The tumours used in this investigation have been mainly the Rous sarcoma and the chemically induced GRCH 15 fowl tumour (Peacock, 1940). A number of human tumours obtained at biopsy was also examined. After excision they were conveyed at once to the laboratory, and were examined immediately by the same methods as were used for the fowl tumours.

Tumour-bearing fowls were killed by decapitation and the blood from the carotid arteries collected in a vessel containing oxalate. The tumour tissue was immediately excised and dealt with as follows :

(*a*) A small portion was fixed for histological examination.

(*b*) Moisture was determined by drying weighed portions in tared beakers at 100° to constant weight.

(*c*) Nucleotide, nucleoside, and free purine N were determined in trichloracetic acid extracts by the method of Kerr and Blish (1932) as modified by Kerr (1940). The sum of all three gave the total acid-soluble purine N. Nucleotides were determined in blood by pipetting 5 ml. blood into 20 ml. 10 per cent. trichloroacetic acid, centrifuging, and following the method of Kerr (1940).

(*d*) The total nucleic acid content of the tissues was estimated on 20–80 mg. portions by making nucleoprotein phosphorus (NPP) determinations by the method of Berenblum, Chain and Heatley (1939).

(*e*) Polypeptide estimations were carried out on tissue by a modification of the method of Burstein (1937). Portions of about 3 g. were weighed out and ground in a mortar with 5 vols. 5 per cent. trichloroacetic acid. After 15 minutes the suspension was filtered. Of the filtrate 2 ml. were taken for total nitrogen determination. 5 ml. were treated with 1 ml. 5 per cent. phosphotungstic acid in 2 N HCl. After 15 minutes the precipitate was centrifuged down, washed with 2 ml. 1 per cent. phosphotungstic acid in 0·4 N HCl and transferred to a digestion flask for total nitrogen determination (micro-Kjeldahl, modification of Ma and Zuazaga, 1942).

For blood polypeptide determinations 5 ml. oxalated blood were laked in a centrifuge tube containing 5 ml. water; 5 ml. 15 per cent. trichloroacetic acid were added. The mixture was centrifuged and the supernatant fluid treated as for tissue extracts.

(*f*) The remainder of the fresh tissue was minced, dehydrated with several successive portions of alcohol, then ether and dried. The dried material was transferred to a stoppered 50 ml. centrifuge tube and shaken for 4 one-hour periods with successive portions of about 40 ml. 0·1 N HCl. This process removes acid-soluble P, including simple nucleotides. The residue was washed twice with alcohol, and was then extracted for two successive two-hour periods at 65° under a reflux condenser with an alcohol-chloroform mixture (3 : 1). It was then washed with ether, dried, ground to a fine powder in a mechanical mortar and passed through a sieve (120 mesh).

Total P was estimated in the extracted powder by a modification of the method of Allen (1940).

The amounts of ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) were determined in the extracted tissue powders by the method previously described (Davidson and Waymouth, 1944*a* and *b*).

The nucleic acids were extracted from the tissue powder with 10 per cent. NaCl. From the extract they were precipitated as lanthanum salts, which were finally decomposed with sodium carbonate. The lanthanum carbonate was centrifuged down, and pentose and desoxypentose were determined colorimetrically in the supernatant fluid. Pentose was estimated by a modification of the orcinol method of Mejbaum (1939), and desoxypentose by a modification of the diphenylamine method of Dische (1930) and Sevag, Smolens and Lackman (1940). Calibration curves were prepared for pentose from liver or yeast ribonucleic acid and for desoxypentose from thymus desoxyribonucleic acid, and were drawn up in terms of RNAP or DNAP.

RESULTS.

The results of the water and NPP estimations are shown in Table I. In nearly all cases the water content of the tumours is high in comparison with normal tissues. The high moisture content of rapidly growing tissues (Cramer, 1916; Willmer, 1935) such as embryonic tissues (Davidson and Waymouth, 1944*a*) and tumour tissues

TABLE I.—*Nucleoprotein Phosphorus (NPP) and Water Content of Tumours. In the Case of Fowl Tumours the Mean Values and the Standard Error are shown and the Number of Tumours Examined is given in brackets.*

	Water content. %	Nucleoprotein phosphorus (N.P.P.) mg. per 100 g.	
		Wet weight.	Dry weight.
Fowl tumours:			
GRCH 15	83·2 ± 0·56 (5)	88·8 ± 2·38 (5)	531 ± 22·6 (5)
Rous sarcoma	88·1 ± 3·10 (12)	53·1 ± 5·38 (12)	462 ± 49·5 (12)
Adjacent muscle	77·0 ± 1·26 (4)	30·5 ± 5·61 (4)	133 ± 23·9 (4)
Rat tumour:			
Fibroadenoma of mammary gland	84	40	247
Human tumours:			
Lymphosarcoma	85	72	490
Fibrosarcoma (thigh)	84	98	529
Sarcoma (breast)	82	72	400
Adenosarcoma (breast)	84	22	140
Carcinoma (breast)	75	63	250
,, (breast)	64	99	280
Secondary carcinoma of colon	82	25	135
Hypernephroma	81	63	335
Carcinomatosis (omentum)	88·5	27	233

(*cf.* Marvelli, 1930; Cavina, 1931) is well known. The NPP figures indicate a high nucleic acid content in the fowl tumours. This is most striking in the case of the NPP values on a dry weight basis owing to the high water content of the tumours. Figures for the NPP content of other fowl tissues on a dry weight basis are heart 161, liver 408, brain 119, nerve 205 mg. per 100 g. (Davidson and Waymouth, 1944*a*). Muscle adjacent to the tumours gave a figure of 133 ± 23·9 mg. per 100 g.

On the other hand, the figures for the NPP content of human tumours vary greatly according to the type of tumour in question. Comparison with the tissue of origin is in most cases not possible, but, in any case, the data presented do not justify any broad generalization about the nucleic acid content of tumours. Stowell (1942) has shown that the (desoxyribo) nucleic acid content may vary greatly even amongst different samples of the same type of tumour.

The nucleotide content of the tumours is shown in Table II, and comprises mainly

TABLE II.—*Nucleotide N, Nucleoside N and Free Purine N of Tumour Tissue. The Sum of all three gives Total Acid-soluble Purine N. In the case of Fowl Tumours Mean Values and the Standard Error are given. The figure in brackets is the Number of Animals.*

	Milligrams per 100 g. fresh tissue.		
	Nucleotide N.	Nucleoside N and free purine N.	Total acid-soluble purine N.
Fowl tumours :			
GRCH 15	19·7 ± 1·38 (4)	9·3 ± 0·45 (4)	29·0 ± 1·16 (4)
Rous sarcoma	10·4 ± 0·92 (12)	7·4 ± 0·37 (12)	17·8 ± 1·18 (12)
Adjacent muscle	43·9 ± 4·42 (8)	11·0 ± 0·51 (8)	55·0 ± 4·18 (8)
Rat tumour :			
Fibroadenoma of mammary gland	7·0	6·3	13·3
Human tumours :			
Lymphosarcoma	9·0	18·5	27·5
Hypernephroma	16·1	9·8	25·9
Carcinoma of colon—secondary growth	11·2	9·8	21·0
Fibrosarcoma of thigh	21·0	12·6	33·6
Carcinomatosis (omentum)	4·9	9·1	14·0

adenylic acid and adenosine triphosphate. In the case of the fowl tumours, especially the Rous sarcoma, the nucleotide concentration is very low. The figures are lower than those found for any adult fowl tissue (Davidson and Waymouth, 1944*a*), and are comparable with those for chick embryonic tissue. The total acid-soluble purine N is also low. The nucleotide concentrations in human tumours are variable, but in most cases they are low as in fowl tumours.

It will be seen from Table III that tumour tissue, like normal tissues, contains appreciable amounts of ribonucleic acid. Of the total amount of phosphorus in the extracted tumour powder some 50–70 per cent. is recovered from the lanthanum

TABLE III.—*Ribonucleic Acid Phosphorus (RNAP) and Desoxyribonucleic Acid Phosphorus (DNAP) as a Percentage of the Total P Recovered from the Precipitate of Lanthanum Salts.*

Type of tumour.	RNAP as % total P.	DNAP as % total P.	RNAP + DNAP as % total P.	$\frac{\text{RNAP}}{\text{DNAP}}$.
Fowl tumours :				
Rous sarcoma (*a*)	73·2	18·3	91·5	4·0
,, ,, (*b*)	73·2	14·7	87·9	4·9
,, ,, (*c*)	85·2	13·4	98·6	6·4
,, ,, (*d*)	77·2	20·6	99·8	3·8
GRCH 15 (*a*)	80·4	15·3	95·7	5·3
,, (*b*)	75·5	15·4	91·0	4·9
,, (*c*)	80·5	18·0	98·5	4·5
,, (*d*)	88·5	15·5	104·0	5·7
Human tumours :				
Lymphosarcoma	50·9	35·7	86·5	1·4
Carcinoma of cervix	58·2	31·5	90·0	1·9
,, of breast	66·4	35·0	101·4	1·9
,, of breast (secondary in axillary lymph node)	49·0	41·4	90·5	1·2
Sarcoma of breast	77·4	22·4	99·8	3·5
Adenosarcoma of breast	65·7	29·2	95·0	2·3

nucleates. Of this, between 90 and 100 per cent. in most cases can be accounted for in terms of RNAP and DNAP. Although it cannot be assumed that the ratios found for the lanthanum precipitates necessarily represent the actual proportions in the original tissue, it does appear that the amount of ribonucleic acid, especially in the fowl tumours, is considerably greater than the amount of desoxyribonucleic acid. In most of the human tumours probably more than 50 per cent. of the total nucleic acid is present in the form of ribonucleic acid.

On account of the presence in many tumours of substances which interfere with the colorimetric procedures, the method of direct estimation of pentose and desoxypentose, which has been used for extracted liver powders (Davidson and Waymouth, 1944*b*), is not generally applicable to tumour tissue. When these have been carried out on the fowl tumours the RNAP/DNAP ratios obtained have been rather lower, as in the case of liver tissue, than those obtained by the indirect method.

Ribonucleic acid has been isolated from Rous sarcoma tissue by a method similar to that employed in the isolation of ribonucleic acid from liver (Davidson and Waymouth, 1944*c*). The tumour tissue was dehydrated with ethanol and the nucleic acids extracted from the dry powder with 10 per cent. NaCl, precipitated with ethanol, and purified through the barium salts (Jorpes, 1934). The ribonucleic acid was finally precipitated from glacial acetic acid and washed with ethanol and ether. Reprecipitation from glacial acetic acid did not render the material completely free from desoxyribonucleic acid. It was protein free, and acted as a substrate for crystalline ribonuclease.

The results of the polypeptide estimations are shown in Table IV. The method employed is not absolutely specific for polypeptides, but is a measure of the amount of nitrogenous material precipitated by phosphotungstic acid, but not by trichloroacetic acid. Nucleotides and nucleosides, for which figures are quoted in the table, are not

TABLE IV.—*Polypeptide Nitrogen, Non-protein Nitrogen (NPN), Nucleotide N, Nucleoside N and Free Purine N, and Total Acid-soluble P of Fowl Blood, Plasma and Tissues. All figures are in milligrams per 100 ml. Blood, Plasma or Tissue.*

Bird number.	Tissue.	Polypeptide N.	N.P.N.	Nucleotide N.	Nucleoside and free purine N.	Total acid-soluble P.
Normal fowls :						
1.	Whole blood	25·2	62·0	5·3	2·8	—
2.	,, ,,	12·1	43·2	—	—	—
3.	,, ,,	13·9	49·3	—	—	—
4.	,, ,,	11·2	42·8	—	—	—
5.	,, ,,	8·9	34·0	—	—	—
6.	,, ,,	7·7	44·8	—	—	—
7.	,, ,,	9·8	47·0	—	—	—
8.	,, ,,	9·2	47·2	—	—	—
9.	,, ,,	13·6	44·3	—	—	—
10.	,, ,,	10·1	40·9	—	—	—
11.	,, ,,	13·5	59·9	—	—	—
	Mean	12·3	46·9	—	—	—
	Standard error	±1·43	—	—	—	—
Fowls with Rous sarcoma :						
1.	Whole blood	23·5	46·2	3·4	5·5	—
2.	,, ,,	28·6	66·1	3·4	5·9	—
3.	,, ,,	16·8	42·0	1·3	5·8	—
4.	,, ,,	23·1	56·6	1·3	8·8	—
5.	,, ,,	16·0	66·6	5·3	4·6	—
6.	,, ,,	13·4	50·2	7·0	6·5	—
7.	,, ,,	19·3	57·8	6·7	3·5	—
8.	,, ,,	14·3	45·2	4·6	4·2	—
9.	,, ,,	13·4	52·5	—	—	—
10.	,, ,,	13·0	57·8	6·7	3·5	—
11.	,, ,,	16·2	54·5	5·6	5·6	—
12.	,, ,,	23·9	60·9	4·9	3·9	—
	Mean	18·5	54·9	—	—	—
	Standard error	±1·55	—	—	—	—
5.	Plasma	5·5	22·1	—	—	—
7.	,,	6·3	26·2	—	—	—
10.	,,	8·4	24·2	—	—	—
1.	Sarcoma	37·4	64·1	14·0	9·8	74·0
2.	,,	21·4	24·1	13·3	9·8	53·5
3.	,,	14·7	46·2	7·0	5·6	36·5
4.	,,	29·4	54·6	14·0	7·7	62·0
5.	,,	19·8	55·7	—	—	—
6.	,,	18·1	47·3	—	—	—
1.	Muscle (breast)	29·8	137·5	35·0	13·3	154·5
2.	,, ,,	81·9	254·0	58·1	10·5	201·5
3.	,, ,,	81·5	261·5	58·8	9·8	185·0
4.	,, ,,	14·3	56·6	58·1	9·1	183·0

precipitated by phosphotungstic acid under the conditions employed, but are included in the NPN fraction.

The figures for the polypeptide content of the blood of both normal and tumour-bearing fowls vary over a wide range. In the case of the tumour-bearing birds the mean value is, however, higher than that for normal fowls and the difference is statistically significant. Both the non-protein nitrogen and the polypeptide nitrogen of the blood of normal and tumour-bearing fowls are high in comparison with normal mammalian blood, where the polypeptide content is of the order of 4 mg. polypeptide N per 100 ml. for man (Arduino, 1941) and for the dog (Burstein, 1937). Comparison of the figures for blood and plasma polypeptide shows that the bulk of the polypeptide N is in the cells. This holds also for mammalian blood (Larizza, 1937; Arduino, 1941; Burstein, 1937; and Claudatus, 1939).

The polypeptide content of the Rous sarcoma tissue is similar to that of whole blood and is much lower than that of muscle.

TABLE V.—*Fibromyomata of the Uterus.*

		Milligrams per 100 g. tissue.				
	Water content. %	Nucleoprotein phosphorus (N.P.P.)		Nucleotide N.	Nucleoside N + free purine N.	Total acid-soluble purine N.
		Wet wt.	Dry wt.			
1. Myometrium	79	52	250	8·8	10·5	19·3
Fibromyoma	83	57	330	8·1	6·7	14·8
2. Myometrium	82	55	310	10·9	10·5	21·4
Fibromyoma	71	114	390	10·0	8·8	18·8

In two cases fibromyomata of the uterus have been compared with the uterine tissue in which they were growing (Table V). Such a comparison is not altogether free from objection, but in both cases the nucleic acid content is higher and the nucleotide content slightly lower in the tumour than in the normal myometrium.

DISCUSSION.

The Rous sarcoma is derived from connective tissue (macrophage or fibroblast, Berenblum, Chain and Heatley, 1940), but it is not feasible to obtain a large enough amount of sufficiently pure connective tissue for comparison of its composition with that of the tumour. On a dry weight basis the fowl tumour tissue has a high nucleic acid content, comparable indeed with that of normal liver. Even higher nucleoprotein phosphorus contents have been found in the Jensen rat sarcoma [e.g. 179 mg. per 100 g. wet weight (Boyland, 1932) or 680 mg. per 100 g. dry weight (Berenblum, Chain and Heatley, 1939)]. Rondoni (1941) found figures of 350 mg. per 100 g. (dry weight) for rat sarcoma and 63 mg. per 100 g. (dry weight) for rat skin and subcutaneous tissue. Liver is one of the few tissues where comparison of tumour with the tissue of origin is possible (*cf.* Burk, 1942). The rat hepatoma (induced by feeding p-dimethylaminoazobenzene) has a higher nucleic acid content than normal rat liver (Dickens and Weil-Malherbe, 1943; Fujiwara, Nakahara and Kishi, 1937; Davidson and Waymouth, 1944*b*). While therefore it may be justifiable to conclude that rapidly growing cellular tumours have, in general, like embryonic tissues, a high nucleic acid content, it is obvious from the figures obtained from the human tumours that this does not hold in all cases. Where a tumour contains large amounts of fibrous tissue, for example, the nucleic acid content, as might be expected, is not high.

The results of the pentose estimations on the lanthanum precipitates indicate that a considerable proportion of the total nucleic acid appears to be ribonucleic acid, and this conclusion has been verified by the isolation of ribonucleic acid from Rous sarcoma tissue. The evidence hitherto adduced for the presence of ribonucleic acids in tumour tissues, e.g. by Caspersson and Santesson (1942) and by Koller (1943*a*, *b*), has been indirect, and the ribonucleic acid of the phospholipin-ribonucleoprotein complexes in the Rous sarcoma virus (Claude, 1939) has not been obtained in sufficient amounts for complete characterization.

Tumour tissue, like embryo tissue, is peculiar in its low acid-soluble nucleotide content. Barrenscheen and Peham (1941) found low figures for the nucleotide content of a miscellaneous collection of tumours. The high amounts of nucleoside relative to nucleotide shown in their tables suggest that the tissues employed were not absolutely fresh. Tumour tissue contains active nucleotidases (Adler and Euler, 1940), such as are present in normal tissues (Reis, 1937), which rapidly convert nucleotides to nucleosides. For that reason all the human material used in this investigation has been freshly obtained at biopsy and post-mortem specimens have been avoided.

The total acid-soluble P (Table IV), which includes nucleotide P, is also much lower in the Rous sarcoma than in fowl muscle, for which the figures are of the same order as those quoted by Boyland (1932) for frog muscle. The polypeptide level in normal fowl blood is considerably higher than in normal mammalian blood, but has been found to be significantly lower than in the blood of tumour-bearing fowls. This is of some interest in view of the statement by Winzler and Burk (1943) that the blood of tumour-bearing rats contains 20–50 mg. per cent. proteose N (precipitable by phosphotungstic acid, but not by trichloroacetic acid), whereas the figure for normal rat blood is 3–4 mg. per cent. In human patients with carcinoma high blood polypetide levels have been reported (Larizza, 1937; Godfried, 1939; Ramond and Zizine, 1922; Hahn, 1921*a*, *b*). The nucleotide level in the blood of our tumour-bearing fowls was normal.

It can be concluded that the materials absorbing light at 2600 Å observed by Caspersson and Santesson (1942) and Caspersson, Nyström and Santesson (1941) in the cytoplasm of rapidly growing tumours and classified as "ribonucleotides" are ribopolynucleotides rather than simple acid-soluble nucleotides. The material observed by Mitchell (1942, 1943) in the cytoplasm of tumour-cells exposed to X- and gamma radiation would appear to be of a similar nature. While tumour tissue contains ribonucleic acid, there appears to be no reason to conclude that the ribonucleic acid content is higher than in many normal tissues, and the presence of ribonucleic acid is certainly not limited to malignant, or even to rapidly growing, tissues. Where the tumour tissue is highly cellular, the total nucleic acid content, including ribonucleic acid, is high. In this respect, and in the low concentrations of acid-soluble nucleotides, tumour tissues resemble the highly cellular tissues of the rapidly growing embryo.

SUMMARY.

Tumour tissue from the Rous sarcoma and from the GRCH 15 fowl tumour has a high water content and a high total nucleic acid content. In a series of human tumours the water content is high, but the total nucleic acid content is variable. In both fowl and human tumours concentrations of acid-soluble nucleotides are lower than in most normal tissues.

Of the total nucleic acid a large proportion is present in the form of ribonucleic acid.

In the blood of fowls with the Rous sarcoma the concentrations of polypeptide N

and of non-protein N are higher than in normal fowl blood. The levels of both are high in comparison with normal mammalian blood.

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SENSITIZATION TO "TETRYL."

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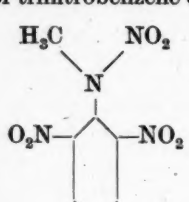
DURING the present war there has been a high incidence of contact dermatitis among workers in ordnance factories, particularly among those engaged in handling the explosives "tetryl" (trinitrophenylmethylnitramine) and fulminate of mercury. The present investigation of the mechanism of skin sensitization to these substances was undertaken in the hope of devising a method for diminishing the considerable loss of labour-time involved. This paper describes experiments with "tetryl" which, although they have not led to a solution of the practical problem of protection against sensitization, have revealed some points of general interest in relation to skin sensitivity. With fulminate no more has been done so far than to show that it is a powerful sensitizing agent for guinea-pigs when given by the intradermal route.

"Tetryl" falls into the class of trinitrobenzene explosives—



"Tetryl,"

which includes picric acid (lyddite) and trinitrotoluene (TNT). Both these are liable to sensitize the skins of those exposed to them, but do not do so nearly as frequently as does "tetryl." "Tetryl" presents in fact a contrast to TNT in that its systemic toxicity is inconsiderable (Cripps, 1917; Wells, Lewis, Sansum, McClure and Lussky, 1920; Noro, 1941; Horner, 1942; Witkowski, Fischer and Murdock, 1942) and its sensitizing potency high, whilst the reverse is true of TNT. It is not a direct irritant to the skin, although it is very irritating to the eyes, probably for physical reasons. Workers in the factories are exposed to direct skin-contact while handling the substance, and to inhalation of the dust; in spite of all precautions the skins of these workers are almost always stained, and the proportion of workers not previously exposed who become sensitized may be as high as 30 per cent. The "incubation period" for sensitization is about 10–14 days. Preventive measures aim at minimizing contact by reducing direct handling as far as possible, by diminishing dust in the shops by proper ventilation, and by the use of non-fatty barrier creams; and workers are instructed to remove all traces after work by washing with a 1 per cent. sulphite soap which decomposes "tetryl," giving a bright red colour. The only reliable method is to

take permanently out of contact all workers who show any signs of sensitivity, and only to employ those who are found by experience to be resistant. This is the course usually followed in peace-time, when the shortage of labour is not a factor. (Schwartz, 1944.)

The present investigation describes experimental methods of sensitizing guinea-pigs to "tetryl," together with a study of the chemical grouping in the molecule of "tetryl" which is responsible for its sensitizing properties, and some experiments bearing on the relation of skin sensitivity to other immune reactions.

EXPERIMENTAL.

Methods of Sensitization of Guinea-pigs.

Five methods of sensitization were used: (1) Intradermal injection; (2) application to a scalded area; (3) subcutaneous implantation of capsules; (4) inunction in a lanoline base; (5) inhalation of a "smoke." Ideally, the method used should reproduce as closely as possible the conditions under which sensitization takes place naturally in the factory. On this basis methods (1), (2) and (3) are not "natural," but were used in most of the work to be reported because, with (1) and (2) at least, good sensitization of a high proportion of the animals was achieved. Method (4) was used because lanoline is known to assist the absorption of fat-soluble substances, and because of the clinical observation that people with greasy skins seemed more liable to "tetryl" dermatitis. It was, however, unsuccessful. Method (5) produced a marked skin-reaction in only one of the eight animals used, but six of these animals were anaphylactically sensitive (detailed results in Table I and Fig. 1); these results may be of significance as regards normal conditions of sensitization of workers.

Albino or cream and white guinea-pigs, weighing 180–220 g. at the beginning of sensitization, were used throughout.

(1) *Intradermal injection.*—0·01 c.c. of M/100 solution of "tetryl" in propylene glycol was injected intradermally into the skin of the flank six times, in the course of a fortnight. After a further week the animals were tested by the application to the epilated skin of the belly of a throat swab dipped in the test solution, usually M/100 in acetone or dioxan. The area covered had a diameter of about 1 cm., and a dozen or more different solutions could be tried on the same animal without difficulty. Twenty-four hours later the animals were examined; reactions were read as follows:

++ Very marked erythema, or oedema.
+ Marked erythema.
+− Slight definite erythema.
tr. Trace or doubtful reaction.

If necessary the area tested was cleaned with acetone to remove the stain of the test solution and facilitate reading. All test solutions were also painted on several control animals to ensure that they would not act as direct irritants.

This method was successful in sensitizing almost all of the animals used. A control group treated with injections of propylene glycol alone showed no sensitization under these conditions.

If animals sensitized by this method (or method (2)) were kept for re-testing, their sensitivity was maintained by a weekly or, better, a fortnightly injection of "tetryl" made up as in (1) ("maintenance dose"). They were not usually tested until at least 7 days after the last maintenance dose; sensitivity seemed to be highest about the tenth day. Small immature animals were used, since they seemed to be more readily sensitized and to give stronger skin-reactions. Once the animals were

TABLE I.—*Skin and Anaphylactic Reactions of Animals Sensitized by Inhalations of Tetryl "Smoke."*

Guinea-pig number.		16.v.44. Skin sensitivity. "Tetryl."	T.N.P.*	Anaphylaxis tests.				
Females	1	tr.	tr.	+−			18.v.44	Dale *in vitro* technique.
	2	,,	,,	+			,,	
	3	,,	−	−			16.v.44	
	4	+−	+−	+			,,	
Males	5	−	−	+	1¼ mins.	(mild symptoms)	18.v.44	Intravenous technique.
	6	tr.	tr.	+++	1¼ mins.	(died 3 mins.)	16.v.44	
	7	,,	,,	−		(discomfort only)	18.v.44	
	8	,,	,,	+	1¾ mins.	(mild symptoms)	16.v.44	

All the animals received 6 ½-hour exposures to a "smoke" of "tetryl" over the course of 14 days, starting 21.iv.44.

Technique.—The *in vitro* tests were done with the isolated uterus by the Dale technique; the +, etc., signs represent the intensity of the sensitivity as judged by the amount of antigen (picrylgelatin) needed to provoke the best reaction. None of these reactions were "maximal," i.e. gave contractions equal to those provoked by an optimal dose of histamine. The males were tested by injecting 0·1 c.c. of picryl-gelatin into the vein of the thigh, which was exposed by a small cut made under local anaesthesia. Reactions were read as follows:

\+ Mild definite symptoms; several coughs, and laboured breathing.
++ Severe symptoms with convulsions and recovery.
+++ Severe symptoms with convulsions followed by death.

The *time of onset* of symptoms after the completion of the injection follows the + signs.

* In this and following Tables T.N.P. = trinitrophenetole.

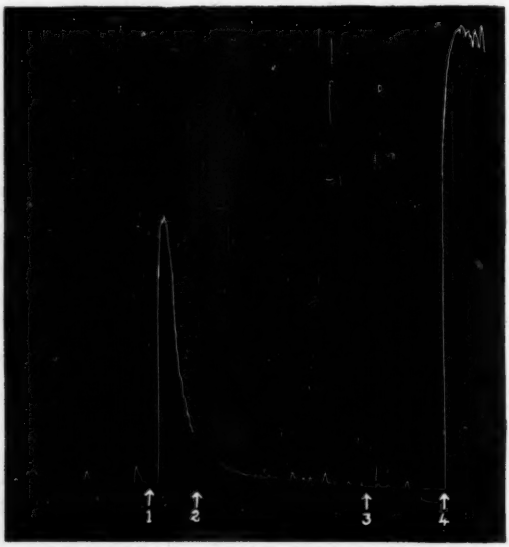


FIG. 1.—Uterine reaction (one horn) of guinea-pig sensitized by inhalation of "tetryl." 1. Antigen No. 28 (picrylgelatin); $1/10^3$ dilution. 2. Wash. 3. Antigen No. 28; $1/10^3$ dilution. 4. Histamine (sufficient to give maximal reaction).

sensitized, fortnightly maintenance doses were sufficient to preserve their sensitivity indefinitely, although the reactions on adult guinea-pigs were weaker, being seldom more than +.

(2) *Subcutaneous implantation of pellets.*—Pellets of " tetryl " weighing 10 or 25 mg. were made in a tablet-machine. Under light ether anaesthesia a cut was made through the skin of the shaved flank, and the capsule pushed with forceps laterally into the subcutaneous tissue until it could be palpated ½ in. to 1 in. from the incision. Collodion was then applied to the incision, which always healed rapidly. The capsule was removed on the fourteenth day by cutting over the point where it could be palpated and squeezing it out, the incision being again closed with collodion. As far as could be judged by the loss of weight of the pellet, absorption from both sizes amounted to 2–4 mg. After a further week the animal was tested as in (1). This method also sensitized nearly all animals, giving slightly more ++ reactions than method (1).

(3) *Scald method.*—The skin of the lower part of the flank was scalded by applying lightly for 1½ seconds the base of a cylindrical aluminium pot (diam. 1 in.) containing boiling water. M/10 " tetryl " in acetone was then applied on a swab to the scalded area, and this was repeated on the second and fourth days. At the beginning of the second week the scalding and applications were repeated on the other side ; the animals were tested as in (1) after a further week. In a small series sensitization was absent or doubtful, and the method was not pursued.

(4) *Inunction.*—A 1 per cent. suspension of " tetryl " in lanoline was made up, and approximately 0·1 g. of this was rubbed well into the epilated skin of the belly. Six treatments over the course of a fortnight were given, and the animals tested as in (1) after a further week. In the small group of animals used none showed any sensitization.

(5) *Inhalation of a " smoke."*—Eight animals were put in a chamber of 580 l. capacity (as described by Campbell, 1936) and exposed for 30 minutes to an artificial smoke of " tetryl " particles, made by blowing compressed air through a 10 per cent. solution of " tetryl " in acetone.* A rough colorimetric analysis of the air showed a concentration of about 0·4 mg. "tetryl " per litre ; the maximum particle size can be calculated as about 2–3μ. Six treatments were given ; the average ventilation of animals of the size used (250 g.) is about 6–8 l. per hour, and so each animal should have absorbed in all about 7–10 mg. Results are shown in Table I.

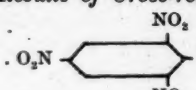
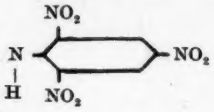
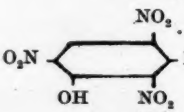
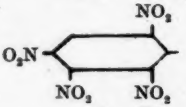
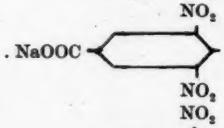
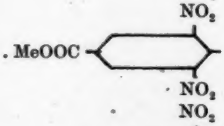
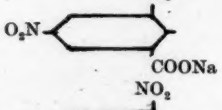
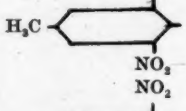
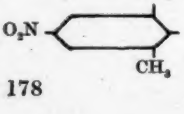
Cross-reactions.

A satisfactory method of sensitization to " tetryl " having been found, it became possible to study the nature of the group responsible. It is generally assumed that simple chemical substances which cause sensitization do so by virtue of their power to couple with proteins, thus producing antigens, after introduction into the body. In the case of " tetryl " it was at first thought probable that the sensitizing antigen would contain the reactive methylnitramino group ; this would imply coupling of " tetryl " with protein through one of its other functional groups. It is known that γ-trinitrotoluene condenses readily with amino-acids by elimination of a nitro group (Barger and Tutin, 1918), and a similar reaction with " tetryl " seemed not unlikely. If this were so, derivatives of " tetryl " in which one of the nitro groups was replaced by a different group, or in which one of the free positions of the benzene ring of " tetryl " was occupied by a substituent, should be able to elicit a skin reaction in " tetryl "-sensitized guinea-pigs. A number of such compounds were prepared and tested with completely negative results, except for a minor reaction with ethoxy-" tetryl " (Table II).

It was then discovered that the " tetryl "-sensitized guinea-pigs reacted strongly to picryl chloride and to 2 : 4 : 6-trinitrophenetole, i.e. to two compounds which

* Apparatus for this experiment was kindly prepared by Mr. J. E. Lovelock.

TABLE II.—*Results of Cross-reactions.*

"Tetryl"*		$-N(NO_2).CH_3$	+++
Picric acid* or its Na salt	„	$-ONa$ or OH	−
Trinitrobenzene (symm.)	„	$-H$	−
Trinitrotoluene (symm.)	„	$-CH_3$	−
Trinitrobenzoic acid (symm.) Na salt	„	$-COONa$	−
Trinitrobenzoic acid (ethyl ester)	„	$-COOEt$	−
Picrylglycine* (neutralized c. NaOH)	„	$-N(H)CH_2.COONa$	−
Picrylglycine (ethyl ester)	„	$-N(H)CH_2.COOEt$	+
Trinitrophenetole*	„	$-OEt$	+++
Picramide	„	$-NH_2$	+
Methyl picramide	„	$-N(CH_3)H$	++
Dimethyl picramide	„	$-N(CH_3)_2$	+
Picryl chloride	„	$-Cl$	++
Dipicrylamine	„		−
Hydroxy-"tetryl"*		$N(NO_2)CH_3$	−
Ethoxy-"tetryl"*		„	+−
Nitro-"tetryl"		„	−
"4-acid"* (neutralized)		„	−
"4-acid" (Me ester)		„	−
"2-acid"* (neutralized)		„	−
"4-Methyl compound"		„	−
"2-Methyl compound"		„	−

contained easily replaceable groups in the position corresponding to that of the methylnitramino group of " tetryl " itself. This directed attention to the alternative possibility that " tetryl " might couple with the amino groups of a protein by elimination of the methylnitramino group, the lability of which is indicated by the ease of hydrolysis of " tetryl " to give picric acid and by the reaction of " tetryl " with aniline to give 2 : 4 : 6-trinitrophenylaniline (van Romburgh, 1890). A renewed study of cross-reactions in the light of this idea showed that the power to elicit skin reactions in " tetryl "-sensitized guinea-pigs extended to picramide and its *N*-methyl and -dimethyl derivatives and to ethyl *N*-2 : 4 : 6-trinitrophenylaminoacetate ; it was lacking in *sym*-trinitrobenzene itself and in derivatives thereof substituted in the 1-position with groups which could not take part in coupling with proteins (Table II).

It thus appeared in general that compounds themselves containing the *N*-2 : 4 : 6-trinitrophenylamino group or potentially capable of forming such a grouping by combination with the amino groups of protein shared the property of eliciting the skin reaction, from which it could be deduced that this group was the essential determinant in the antigen formed by combination of " tetryl " with proteins in the manner already indicated.

It must be noted that two exceptions have been observed to the above general statement, namely, *N*-2 : 4 : 6-trinitrophenylaminoacetic acid (picrylglycine) and di-2 : 4 : 6-trinitrophenylamine (dipicrylamine) ; no final explanation of the anomalous behaviour of these two compounds can be offered, but it may be noted that they differ from the compounds which gave positive results chiefly in their pronounced acidic character. That the glycine derivative does conform in other respects to the theory is shown by immunological experiments to be described below.

Preparation of Picrylprotein Antigens.

The evidence concerning the nature of the hapten group involved in sensitization to " tetryl " which has been given in the previous section would clearly be strengthened if skin sensitization could be correlated with the formation of other antibodies specifically directed against this group, or if the process of sensitization could be influenced in any way by systemic immunization with an antigen containing such groups as determinants. For the study of both these possibilities it was necessary to prepare derivatives of protein analogous with those supposedly formed by combination with " tetryl," i.e. containing 2 : 4 : 6-trinitrophenyl residues as substituents in their free

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EXPLANATION OF TABLE II.

Solutions in acetone at M/100. Those marked * were also used in solution at M/10, without significantly different results. The + signs in this Table refer to the number of guinea-pigs showing reactions, as well as to their intensity.

Note.—The less familiar of the above compounds were prepared according to the following methods :

Picrylglycine, see K. Hirayama. Hoppe-Seyler's *Z.*, **59**, 290 (1909).

Picrylglycine ethyl ester. 2·35 g. glycine ester was dissolved in 5 c.c. alcohol and added to a solution of 2·5 g. picryl chloride in 30 c.c. alcohol. An oil separated at once which crystallized on scratching. Recrystallized from alcohol ; m.p. 92–93° C. Found (Dumas) : N. 17·7 per cent. $C_{10}H_{11}O_8N_4$ requires N, 17·9 per cent.

2 : 4 : 6-*Trinitro-3-ethoxy-methylnitramine.* See P. van Romburgh, *Rec.*, **8**, 274 (1889).

3 : 5-*Dinitro-4-methylnitraminobenzoic acid.* See F. Reverdin and A. de Luc, *Ber.*, **41**, 502 (1908).

3 : 5-*Dinitro-4-methylnitraminobenzoic acid, methyl ester.* See F. Reverdin and A. de Luc, *op. cit.*

3 : 5-*Dinitro-2-methylnitraminobenzoic acid* was prepared from 2-methyl-amino-3 : 5-dinitro benzoic acid under the conditions used for the preceding compounds (F. Reverdin and A. de Luc, *op. cit.*). The compound crystallized from chlorobenzene in hexagonal plates, m.p. 160° C.

3 : 5-*Dinitro-4-methylnitraminotoluene.* See P. van Romburgh, *Rec.*, **3**, 404 (1884).

3 : 5-*Dinitro-2-methylnitraminotoluene.* See P. van Romburgh, *Rec.*, **3**, 396 (1884).

amino groups. Such artificial antigens were conveniently prepared by the use, not of "tetryl" itself, but of the more reactive picryl chloride.*

Method 1 (Antigen No. 43).—65 c.c. of dialysed rabbit serum containing 2·5 g. of protein were diluted with an equal volume of dioxan and the minimum amount (10 c.c). of 0·5 *N* NaOH to keep the protein in solution. The solution was stirred in a bath at 40° C., and 0·9 g. of picryl chloride dissolved in 8 c.c. of dioxan was added slowly over 1½ hours. Small amounts of 0·5 *N* NaOH were added concurrently to keep the *p*H of the solution alkaline to cresol red. Stirring was continued for a further 20 mins. after the addition of picryl chloride was completed. The solution was dialysed against running tap-water for 15 hours, after which the volume was 210 c.c. The precipitate which had appeared was dispersed with 5 c.c. of 0·5 *N* NaOH and 115 c.c. alcohol added; the protein was then precipitated by the addition of 15 c.c. 2 *M* acetate buffer at *p*H 4·6, and was collected by filtration. It was resuspended in 100 c.c. water, dissolved with 5 c.c. 0·5 *N* NaOH, diluted with 200 c.c. alcohol and re-precipitated with 15 c.c. acetate buffer. This purification was repeated a second time. The product was re-suspended in water, dissolved with the minimum amount of NaOH, and dialysed against running tap-water for two days. A considerable amount of precipitate separated. This was filtered off and suspended in 100 c.c. water. A dry weight determination indicated 2·6 per cent. solid. Colorimetric determination using picryl-glycine as a standard showed that the product contained 3·8 per cent. picryl-groups estimated as picrylglycine. The concentration of protein was adjusted to 2 per cent. and the suspension was used for intramuscular or intraperitoneal injection. A dry weight determination on the filtrate (55 c.c.) gave 0·79 per cent. solid, and of the solid a content of 4·2 per cent. picryl estimated as picrylglycine. This solution was used for intravenous administration.

This general method was used for preparing the antigens for immunization of rabbits. The products were frequently largely insoluble, but nevertheless acted as satisfactory immunizing antigens.

Method of estimating picryl groups.—Standard solutions (made from a stock solution of picrylglycine containing 1 mg. per c.c. in glacial acetic acid) of 0·1, 0·2 and 0·5 mg. were made up to 4 c.c. with glacial acetic acid, 1 c.c. of concentrated HCl was added, and also an amount of plain serum with a protein content approximately equal to that of the solution to be tested. The solution of coupled protein to be tested (0·2 to 5 c.c.) was mixed with 4 c.c. glacial acetic acid and 1 c.c. HCl, and together with the standards was heated for 40 mins. in a boiling water-bath to hydrolyse.

After hydrolysis the tubes were cooled and zinc dust was added in two lots, the first of about 800 mg., and after 3 mins. a further 400 mg. The reduction took in all 5 mins. The solutions were then decanted from the zinc into 10 c.c. graduated cylinders and the zinc washed several times with water, the final volume being made up to 10 c.c. The solutions were filtered into 15 c.c. graduated cylinders and the volumes of filtrate adjusted to 8 c.c. 1 c.c. of 1 per cent. sodium nitrite solution was added and, after 3 mins., 1 c.c. of 5 per cent. sulphamic acid solution. After 2 mins. 2 c.c. of 1·0 per cent. naphthylethylenediamine hydrochloride was added, and the colours were compared after 10 mins.

Method 2.—This method was used for preparing picrylgelatin; some lots of picryl-serum-protein were also made by it (using a lower temperature). The product was always of good solubility, and was used for *in vitro* precipitin tests and for tests on anaphylaxis.

* *Note.*—Both tetryl and trinitrophenetole may be used in this reaction if the pH is kept at 8·5. The potassium salt of trinitrophenetole will also react readily with protein at this pH, and has the additional advantage of being water-soluble. Antigens prepared with any of these compounds give the same sort of reactions with antisera as those prepared with picryl chloride.

Preparation of antigen 28.—2 g. of gelatin (Gold Leaf) were dissolved in 40 c.c. of water at 70°; 0·6 g. of picryl chloride in 10 c.c. of alcohol (warmed to dissolve) was added in ½ c.c. quantities, *N* NaOH being added drop-wise from a pipette to keep the *p*H at about 8·0. The solution was then dialysed overnight, precipitated with a few c.c. of dilute acetic acid and redissolved by warming in 20 c.c. of water, with sufficient alkali to bring the substance into solution. The precipitation and re-solution were repeated twice; after the final re-solution the *p*H was adjusted to 7·4, and NaCl was added to bring the concentration of salt to 0·85 per cent. The concentration of protein was adjusted to 2 per cent. before use. Picryl-groups = 5·0 per cent. as picrylglycine.

Rabbit-antiserum.—In order to have a standard of comparison for the various preparations of antigen, an "anti-picryl-rabbit-serum" antiserum was made. Two rabbits were used; each received, during a seven weeks' course, 18 injections totalling approximately 300 mg. of "picryl-rabbit-serum" (prepared as in Method 1). This was given intravenously when the preparations were sufficiently soluble, otherwise intramuscularly or intraperitoneally. The animals were rested for a week; then a further two weeks' course was given, amounting to 60 mg.; 50 c.c. of blood were taken from the ear on the fifth and seventh days after the last injection. The fortnightly courses and bleedings were continued as long as serum was needed. The ring-test titres of the sera, against an antigen prepared by method 2 with horse-serum, varied between 1/125 and 1/625 of a 1 per cent. solution. The "equivalence point" of a bulked lot of serum was about 1/50 of a 1 per cent. solution. These sera, although not very strong, were quite satisfactory for the purpose.

RESULTS.

The occasional presence of circulating precipitins in sensitive animals has been recorded by Landsteiner and Chase (1937), and Cannon and Marshall (1940) claim to have demonstrated circulating antibody, by their collodion-particle technique, in a number of humans sensitive to egg-albumin or insulin. It has, however, been impossible

TABLE III.—*Effect of Parenteral Immunization of Sensitized Guinea-pigs.*

Date.	Test animals.				Controls.			
G.P. No.:	1.	2.	3.	4.	5.	6.	7.	
13.vii.44 .	250	280	260	320	300	310	350	. Weights (g.).
13.vii.44 to 24.vii.44 .	All received * course of sensitization (·03 c.c. dose)							
1.viii.44 .	+−	+	+	++−	++	+	+	. Sensitivity to "tetryl."
	+	tr.	+	++−	++−	+−	+−	. Sensitivity to T.N.P.
2.viii.44 to 14.viii.44 .	6 intraperitoneal injections of Antigen 65† (0·25 c.c.)				*Nil*			. Course of immunization of test animals.
7.viii.44 .	"Maintenance dose" of "tetryl" to all animals							
21.viii.44 .	++	++	+	++	tr.	−	−	. Serum: precipitins.‡
	+	++	+	+	++	+	++	. Sensitivity to "tetryl."
	+	+	+	++	++	+	+	. Sensitivity to T.N.P.

* In this and subsequent Tables the course of sensitization was as follows: "Tetryl" was made up in propylene glycol in a M/100 (0·29 per cent.) solution; 0·01 to 0·03 (according to weight) of this was injected intradermally 3 times a week for 2 weeks.

† Antigen 65 = "picryl-guinea-pig-globulin": 2 per cent. solution.

‡ In this and subsequent Tables, estimated as follows: A few drops of blood were taken from the ear and the separated serum put up in ring test at various dilutions of antigen, usually 1/5 to 1/625 dilutions of a 1 per cent. antigen. + signs refer both to titre and intensity of reaction. A ++ reaction is one giving a very marked ring at 1/25, a definite sharp ring at 1/125, and sometimes a slight reaction at 1/625.

to demonstrate with certainty, by the ring test (using several dilutions of antigen), the presence of circulating precipitins in these " tetryl "-sensitized animals, although doubtful low-titre results were often given by the sera taken from animals a few days after the end of the initial course of sensitization. Possibly the degree of sensitivity of these animals was not high enough to allow of the appearance of precipitins in the serum ; but it seems more likely, from the results which follow, that demonstrable circulating precipitins are a " by-product," and are not directly relevant to the phenomenon of skin reactivity.

The results obtained using picrylproteins as antigens are detailed in Tables III–IX. Table III shows the effect of a course of intraperitoneal immunization with such antigens upon sensitive animals. It will be seen that the sensitivity is neither significantly increased nor depressed. Tables IV and V show a similar lack of effect of either previous or concurrent immunization on the sensitization process, and demonstrate (Table IV, *a*) the complete lack of correlation between the skin and the precipitin reactions. In several other groups of guinea-pigs parenterally immunized, there was similarly no evidence of skin sensitivity of the " contact " type.

From these results it might be suggested that our picryl antigens have no effect on " tetryl "-sensitized animals, and that the deduction that sensitivity to " tetryl " is dependent on the addition of picryl groups to body-proteins is incorrect. However, results on anaphylaxis, shown in Tables I and VI and Figs. 1–4, put this suggestion out of court. It was found that in almost every case intravenous injection of picryl-antigen (picrylgelatin* in the case of the results shown in Table VI) into sensitized guinea-pigs will produce typical anaphylactic symptoms, frequently leading to death. By the Dale technique, the uteri of sensitive animals give contractions, followed by desensitization, after contact with high dilutions of antigen (Fig. 2, *a*) ; normal uteri are unaffected, even by much larger quantities. Landsteiner and Chase (1937) had similar results with animals sensitized to various simple chemicals, including picryl chloride, and injected intravenously with the appropriate antigenic conjugate, and Fierz, Jadassohn and Stoll (1937) report anaphylaxis in animals previously injected with a diazonium complex.

We may here refer to Table I and Fig. 1, showing the results on guinea-pigs sensitized by inhalation, although they constitute something of a side-issue. These results are of considerable interest from the point of view of the industrial sensitization of humans. Although the skin reactions were not striking, tests for anaphylaxis were definitely positive in 6 out of 8 animals and, since simple skin application of " tetryl " was found to be ineffective in producing sensitivity in an earlier group of guinea-pigs, it is probable that the path of sensitization is *via* the lung ; this gives an additional emphasis to the need for reducing the dust-hazard in factories where chemicals able to sensitize are handled. It is likely that alteration in technique might have produced more striking skin reactions.

Figs. 2, *a* and *b*, show respectively the uterine and intestinal *in vitro* reactions of a sensitized guinea-pig. Fig. 3 (upper tracing) shows the reaction of the uterus of a guinea-pig injected with 0·5 c.c. of the serum of a sensitive guinea-pig ; it should be noted that this serum contained no precipitins demonstrable by the ring-test. Fig. 4 shows a similar passive sensitization effect, this time by the use of the serum of a guinea-pig immunized intraperitoneally with picrylprotein, and not skin sensitive. It may be that the Dale test is very much more sensitive in detecting circulating precipitin-like antibodies than the ring-test ; or possibly that a type of antibody present in sensitive guinea-pigs but not demonstrable as a precipitin is able to sensitize

* This antigen was used as being less likely to produce aberrant results owing to the protein " carrier." Other picrylproteins, including picryl-guinea-pig globulin, are equally effective.

TABLE IV.—*Showing* (a) *Lack of Correlation Between Circulating Precipitins and Sensitivity* (*Cages I and II*); (b) *Failure of Previous Immunization to Affect Sensitization* (*Cages I and III*).

Date.	Cage No. I.				II.				III.							
G.P. No.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	
19.vi.44	205	180	230	190	180	180	185	190	..	..	..	..	..	..	..	Weights (g.).
19.vi.44 to 30.vi.44	6 intraperitoneal doses of Antigen 65*				Course of sensitization				*Nil*							
12.vii.44	−	−	−	−	++	++	++−	++−	..	..	..	..	..	..	..	Skin sensitivity to "tetryl."
„	−	−	−	−	++	++	++−	++−	..	..	..	..	..	..	..	Skin sensitivity to T.N.P.
13.vii.44	++	++	++−	+	−	−	−	−	..	..	..	..	..	..	..	Serum: precipitins.
„	300	300	330	300	..				250	300	300	280	320	260	310	Weights (g.).
13.vii.44 to 24.vii.44	Course of sensitization				..				Course of sensitization							..
31.vii.44	+	+−	+	+	..				+−	+	++	+	++−	+	+	Skin sensitivity to "tetryl."
„	+−	Tr.	+	+−	..				+	+−	++−	tr.	++−	+	+−	Skin sensitivity to T.N.P.
„	390	390	440	400	..				375	440	370	400	430	335	420	Weights (g.).

* Antigen 65 = "picryl-guinea-pig-globulin": 2 per cent. solution.

TABLE V.—*Effect of Concurrent Immunization on Sensitizability.*

Date.	Test animals.					Control animals.					
G.P. No.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	
19.x.43 to 5.xi.43	9 subcutaneous injections of Antigen 27*					*Nil*					..
27.x.43 to 5.xi.43	Course of sensitization for all animals.										
25.x.43	200	255	200	210	210	185	230	205	225	190	Weights (g.).
1.xi.43	+	++	++	+−	++	−	−	−	−	−	Serum: precipitins.
11.xi.43	++	++	++	++	++	?†	−	?†	−	?†	„ „
„	−	+−	tr.	+	−	++	tr.	+−	+	+−	Sensitivity to "tetryl."
„	+−	+	„	+	+	++	+	+	+	+	„ T.N.P.

* Antigen 27 = "picryl-rabbit-serum": 2 per cent. solution.

† Doubtful reactions at higher concentration of antigen, such as are sometimes observed immediately after the course of sensitization.

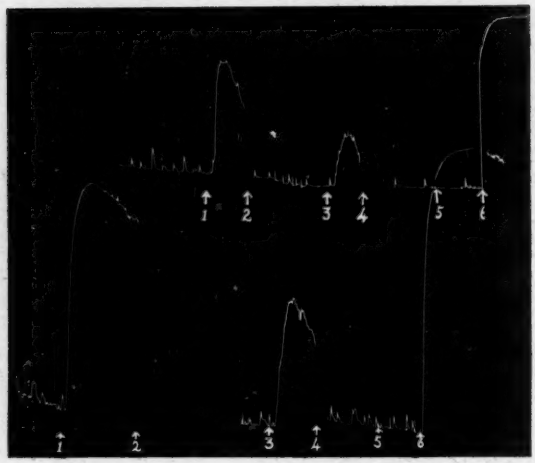

FIG. 2*a*.—Uterine reaction (both horns) of guinea-pig sensitized by intradermal injections of "tetryl." Upper tracing: 1. Antigen No. 28; $1/10^6$ dilution. 2. Wash. 3. Antigen No. 28; $1/10^5$ dilution. 4. Wash. 5. Antigen No. 28; $1/10^5$ dilution. 6. Histamine.

Lower tracing: 1. Antigen No. 28; $1/10^5$ dilution. 2. Wash. 3. Antigen No. 28; $1/10^5$ dilution. 4. Wash. 5. Antigen No. 28; $1/10^5$ dilution. 6. Histamine.

This record shows the difficulty of desensitizing the uterus of a sensitized animal; full desensitization is achieved at 5, in both tracings.

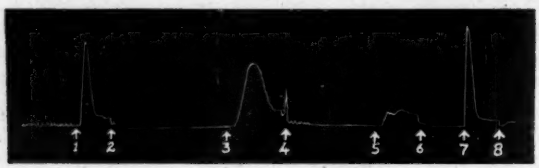

FIG. 2*b*.—Intestinal reaction of guinea-pig sensitized by intradermal injections of "tetryl." 1. Antigen No. 28; $1/10^5$. 2. Wash. 3. Antigen No. 28; $1/10^4$. 4. Wash. 5. Antigen No. 28; $1/10^3$. 6. Wash. 7. Histamine. 8. Wash.

This record shows the difficulty of desensitizing the intestine of a sensitized animal; desensitization is not complete, though increasing amounts of antigen give decreasing reactions. Normal intestine does not react to this antigen even at $1/10^2$ dilution.

uterine tissue, as well as is the ordinary precipitin. Quantitative work on these two methods of passive sensitization is in progress.

Fig. 3 (lower tracing) shows also that picrylglycine, which acts as a powerful inhibitor of the rabbit "anti-picryl" serum, is equally an inhibitor of the uterine reaction. The contraction of the uterus of a strongly sensitized guinea-pig is usually only partially inhibited; the reaction shown is from a normal guinea-pig sensitized rather weakly by intracardiac injection of 1 c.c. of the serum of a sensitive guinea-pig.

Table VI shows, in group (*a*), the failure of large quantities of antigen to elicit any symptoms in normal guinea-pigs, and in group (*b*) the reactions elicited in whole guinea-pigs sensitized to "tetryl." Groups (*c*), (*d*) and (*e*) show results of attempts to inhibit the reaction by various means. It will be seen that desensitization, even with protein-antigen (group (*d*)), is difficult, although one, or better two, subcutaneous

injections previous to the shocking dose exert a definite protective effect. Picrylglycine injected the day before the shocking dose (group (*e*)) has no effect, presumably owing to its rapid excretion, while if it is injected together with the shocking dose (group (*c*)), it seems to exert, as would be expected from the *in vitro* results, a slight protective effect. The number of animals used is, however, rather small.

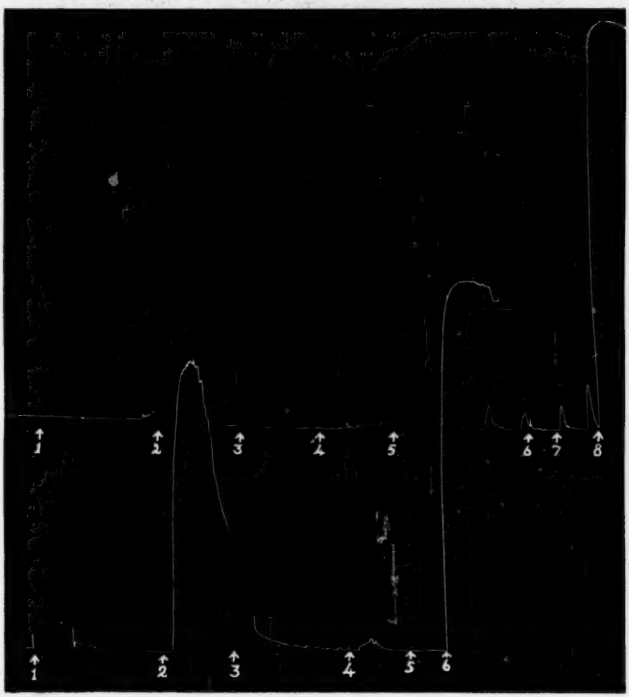

FIG. 3.—Uterine reaction (both horns) of guinea-pig sensitized passively by injection intracardially of 1 c.c. of serum from a "tetryl"-sensitized guinea-pig (showing no precipitins by ring test); also showing inhibition of reaction by "picrylglycine."

Upper tracing: 1. 0·5 c.c. picrylglycine M/20 in saline at *p*H 7·4. 2. Wash. 3. 0·5 c.c. picrylglycine M/20 in saline at *p*H 7·4. 4. Antigen No. 28, $1/10^3$. 5. Wash. 6. Antigen No. 28, $1/10^3$. 7. 0·5 c.c. picrylglycine M/20 in saline at *p*H 7·4. 8. Histamine.

Lower tracing: 1. Wash. 2. Antigen No. 28, $1/10^3$. 3. Wash. 4. Antigen No. 28, $1/10^3$. 5. 0·5 c.c. picrylglycine M/20 in saline at *p*H 7·4. 6. Histamine.

This record shows, in the lower tracing, a good but not maximal reaction to a fairly large dose of antigen, followed by desensitization; picrylglycine is added at 5, and histamine at 6, to demonstrate that picrylglycine does not interfere with normal contractility. The upper tracing shows complete inhibition of contraction in the presence of picrylglycine, followed by desensitization.

Table VII shows the effect on sensitive guinea-pigs of circulating antigen introduced intraperitoneally. There is a transient diminution, but not a suppression, of the skin reaction. Landsteiner and Chase (1937) record similar results. Tables VIII and IX show an interesting contrast between two types of "hapten." Picrylglycine, which is chemically inactive, has no effect whatever on the skin reaction, while trinitrophenetole, which can combine with protein to form a full antigen, has a

TABLE VI.—*Anaphylactic Reactions of Sensitive Animals.*

	Date.	G.P. No.	Sensitivity.	Antigen.	Dose.	Route.	Time (mins.).	Result.
(*a*)	21.2	1	−	45	0·5	I–V	*Nil*	−
Normals, 4;	,,			,,	,,	I–C	,,	−
negative	,,	2	−	,,	,,	I–V	,,	−
controls.	,,	3	−	,,	,,	,,	,,	−
	,,			,,	,,	I–C	,,	−
	,,	4	−	,,	,,	I–V	,,	−
(*b*)	,,	5	+−	,,	0·1	,,	½–4½	D
Sensitive	,,	6	+−	,,	,,	,,	½–3	D
guinea-pigs, 9;	,,	7	+	,,	,,	,,	½–3	D
positive	,,	8	+	,,	,,	,,	3–R	+
controls.	,,	9	+	,,	0·15	I–C	1–5½	D
	,,	10	+−	,,	0·1	I–V	? slight	+−−
	,,	11	+	,,	,,	,,	½–3½	D
	,,	12	+−	,,	,,	,,	½–1	D
	,,	13	+−	,,	,,	,,	½–2	D
(*c*)	,,	14	+	45 : 1 pt. PG : 2 pt.	0·3	,,	1½–R	+
Sensitive	,,							
guinea-pigs, 6.	,,	15	+	45 : 2 pt. PG : 2 pt.	0·4	,,	1½–7	D
Mixture of antigen and picryl-glycine M/20.	,,	16	+−	45 : 1 pt. PG : 2 pt.	0·3	,,	*Nil*	−
	,,	17	+	,,	,,	,,	1½–4	D
	,,	18	+	,,	,,	,,	1½–4½	D
	,,	19	+−	,,	,,	,,	1½–R	++
(*d*)	,,	20	+	45	0·2	S–C		
Sensitive	22.2			,,	0·5	I–P		
guinea-pigs, 10.	23.2			,,	0·1	I–V	3½–R	+ (delayed)
1 or more	21.2	21	+	,,	0·2	S–C		
previous	22·2			,,	0·1	I–V	½–3	D
injections	21.2	22	+−	,,	0·2	S–C		
of antigen	22·2			,,	0·1	I–V	*Nil*	−
(I–P or I–M).	22·2	23	+	,,	0·5	S–C		
	23·2			,,	0·15	I–V	½–3	D
	22.2	24	+−	,,	0·5	S–C		
	23·2			,,	0·1	I–V	? slight	+−
	22.2	25	+	,,	0·5	S–C		
	23·2			,,	0·1	I–V	½–3½	D
	22·2	26	+	,,	0·5	S–C		
	23·2			,,	,,	I–M		
	24.2			,,	0·1	I–V	1½–R	++
	23·2	27	+	,,	0·5	I–M		
	24.2			,,	,,	I–P		
	25.2			,,	0·1	I–V	4½–R	+
	23.2	28	+	,,	0·5	S–C		
	24.2			,,	,,	I–P		
	25.2			,,	0·1	I–V	2–R	+
	22.2	29	+	,,	0·5	I–P		
	23.2			,,	,,	I–M		
	24.2			,,	0·1	I–V	1½–R	++
(*e*)	21.2	30	+	PG	0·5	I–C		
Sensitive	22.2			45	0·1	I–V	1–4½	D
guinea-pigs, 5.	21.2	31	+	PG	0·5	I–C		
1 previous	22.2			45	0·1	I–V	1–2	D
injection	21.2	32	+−	PG	0·5	I–C		
of picryl-glycine M/20.	22.2			45	0·1	I–V	1½–3½	D
	22.2	33	+−	PG	0·5	I–P		
	23.2			45	0·1	I–V	1½–R	++
	22.2	34	+	PG	0·5	I–P		
	23.2			45	0·1	I–V	½–2	D

Analysis of Results.

Group.	Total in Group.	Reaction.			Percentage surviving.	Percentage slightly or not affected.
		Death.	Severe.	Slight or *nil*.		
(*a*) Negative controls	4	0	0	4	100	100
(*b*) Positive controls	9	7	1	1	22	11
(*c*) Mixture of antigen and PG	6	3	1	2	50	33
(*d*) Previous injection of antigen	10	3	3	4	70	40
(*e*) Previous injection of PG	5	4	1	0	20	0

Symbols, etc.

Antigen 45 = "picryl-horse-serum," 2 per cent. solution. PG = picrylglycine, M/20 solution, in saline, buffered at *p*H 7·4. I–V = Intravenous. S–C = Subcutaneous. I–M = Intramuscular. I–C = Intracardiac. I–P = Intraperitoneal. R = Recovery. D = Death.

Col. 3 shows grade of sensitivity, as tested on 20.ii.44.

„ 7 shows time of onset of symptoms and time of death, or recovery.

„ 8 shows intensity of reaction: D = death; ++ = severe, with convulsions; + = definite reaction, coughing and straining.

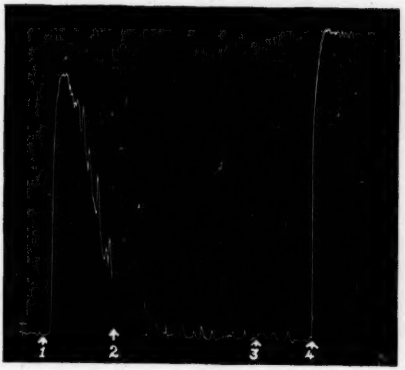

FIG. 4.—Uterine reaction (one horn) of guinea-pig sensitized passively by injection intracardially of 1 c.c. of the serum from a guinea-pig immunized by intraperitoneal injections of picryl-protein (showing precipitins but no skin-sensitivity).

1. Antigen No. 28, $1/10^4$. 2. Wash. 3. Antigen No. 28, $1/10^4$. 4. Histamine.

Note.—The capacity of the bath used for each horn was 15 c.c. The uteri were suspended in Ringer-Locke solution, the intestine (Fig. 2b) in Ringer-Tyrode solution and they were washed with the appropriate solutions. All solutions were kept at 38°C. 0·1 c.c. antigen was added at the dilution indicated.

slight but definite suppressive effect, rather more marked after 72 hours than after 24. It is suggested that a slowly formed "conjugate," rather than the simple hapten, is responsible for this partial suppression, which is thus analogous to the suppression by externally formed protein antigen shown in Table VII.

Finally, Table X shows the result of an attempt to confirm directly the hypothesis that sensitization occurs *via* the production of an antigenic conjugate with tissue protein. The first pair of experiments (Cage 1, test animals, and Cage 2, controls) gave an apparently positive result, but there was a possibility that the antigen used might contain unconjugated picryl chloride, which has an appreciable solubility in water. Therefore a similar antigen (picryl-guinea-pig-globulin) was treated with glycine at an alkaline *p*H in order to remove residual picryl chloride, a precaution taken by Landsteiner and Chase (1941) when demonstrating the possibility of producing

TABLE VII.—*Effect of Circulating Antigen: Temporary Reduction of Sensitivity Reaction.*

Date.	Previously sensitized animals. Test animals. G.P. No. 1.	2.	3.	4.	Controls. 6.	7.	
23.viii.43	+−	−	+	tr.	+−	tr.	Sensitivity to "tetryl."
	+	tr.	++	+−	+−	+−	„ T.N.P.
17.ix.43	tr.	−	+−	tr.	+	tr.	„ "tetryl."
	+−	+	++	+−	+	+	„ T.N.P.
20.ix.43	Intraperitoneal dose (0·5 c.c.) of Antigen 25*				*Nil*		
21.ix.43	−	tr.	−	−	+	tr.	„ to "tetryl."
	−	−	+−	−	tr.	+−	„ T.N.P.
	"Maintenance" injection of "tetryl" to all animals.						
24.ix.43	tr.	tr.	tr.	tr.	+	+−	„ "tetryl."
	„	+−	„	„	+−	+	„ T.N.P.
28.ix.43 to 5.x.43	"Maintenance" injections of "tetryl" to all animals.						
5.x.43	−	−	−	−	−	−	Serum: precipitins.
11.x.43	−	−	−	−	−	−	„ „
	+−	+	+−	tr.	+	+	Sensitivity to "tetryl."
	+	+−	+	+−	+	+	„ T.N.P.

* Antigen 25 = "picryl-rabbit-serum": 2 per cent. solution.

skin sensitization by the intraperitoneal injection of conjugates. A picryl-horse-serum antigen, similarly treated, was also used, to find whether a homologous protein was necessary. It will be seen that Cages 3 and 4 showed poor or negative sensitization, in spite of an increased dosage. This contrast strongly suggests that such sensitization as occurred was produced by the presumably minute amount of picryl chloride still present, and not by the conjugate itself.

The discussion which follows is confined to examples of skin sensitivity of the contact type.

DISCUSSION.

The recent work of the late Karl Landsteiner and co-workers and others has demonstrated without reasonable doubt that skin-sensitivity is an immunological reaction, i.e. that it is the result of the production of specific antibodies. The most likely way for this to happen from both chemical and biological evidence is *via* the production by the excitant of a "conjugate" with the body-proteins of the recipient animal. Such of the above-described results as parallel Landsteiner's support these conclusions.

This work has been directed towards the discrimination between the various types of antibody, namely, of those responsible respectively for the skin reaction, the anaphylactic reaction, and the "precipitin" reaction. In particular it is evident that there is no correlation between demonstrable circulating precipitins and skin reactivity (Tables III–V). Landsteiner and Chase (1940) show that typical skin sensitivity can be produced by the intraperitoneal injection of the excitant together with tubercle bacilli, i.e. that it is not essential that the skin should be the site of fixation of the antigen for skin-sensitivity to occur. In a short but suggestive communication (1942) they claim to have transferred the skin reaction passively by injecting into normal animals the washed centrifuged deposits from the peritoneal exudates of animals sensitized in this way, and suggest that the characteristic cells of such exudates, presumably mainly large mononuclears, carry a special sort of antibody.

TABLE VIII.—*Effect on Sensitivity of Circulating Hapten (Picrylglycine).*

Date.	G.P. No.	Previously sensitized animals. Test animals. 1.	2.	3.	4.	5.	6.	7.	Controls. 8.	9.	10.	11.	12	13.	14.	
23.ix.43	.	+	+	+−	tr.	tr.	+−	+−	+	+−	+	tr.	+−	+−	+	Sensitivity to "tetryl."
		+	+−	+−	+−	tr.	+−	+−	+−	+−	+	tr.	+−	+−	+	„ T.N.P.
26.ix.43	.	Intraperitoneal injection (3·5 mg.) of picrylglycine*										*Nil*				..
27.ix.43	.	+	+	+	+−	+	tr.	+	+	+	+	+	+−	tr.	+	„ "tetryl."
		tr.	+−	+	+−	+−	tr.	+	+−	+−	+	+	+−	+−	+	„ T.N.P.
28.ix.43	.	"Maintenance injection" of "tetryl" to all animals.														.
30.ix.43	.	+	+	+−	−	+	tr.	+−	+	+−	+−	+−	−	+	+−	„ "tetryl."
		tr.	tr.	tr.	−	+	+−	−	+−	tr.	+	tr.	−	+−	+	„ T.N.P.

* M/20 solution buffered at *p*H 7·4.

TABLE IX.—*Effect of Circulating Hapten (Trinitrophenetole).*

Date.	G.P. No.	Previously sensitized animals. Controls. 1.	2.	3.	4.	Skin-test 1 day after injection. 5.	6.	7.	8.	9.	10.	11.	Skin-test 3 days after injection. 12.	13.	14.	
27.ix.43	.	+	+	+	+−	+−	+	+−	+	+	tr.	+	+	+−	tr.	Sensitivity to "tetryl."
		tr.	+−	+	+−	+−	+	+−	+	+−	tr.	+	tr.	+	−	„ T.N.P.
5.x.43	.	"Maintenance" injection of "tetryl" to all animals.														
10.x.43	.	*Nil*				Intraperitoneal injection of 2·5 mg. T.N.P. in prop. glycol							*Nil*			..
11.x.43	.	+	+	+	+−	−	tr.	−	tr.	tr.	+−	+	+−	+	+	Sensitivity to "tetryl."
		+	+	tr.	+−	−	−	−	++	−	−	+	tr.	+	+	„ T.N.P.
12.x.43	.	*Nil*							*Nil*				I-P injection of 2·5 mg. T.N.P. in prop. glycol.			..
15.x.43	.	+−	+	+−	tr.	−	+−	tr.	+	tr.	+−	+	tr.	−	−	„ "tetryl."
		+−	+	+−	tr.	−	tr.	tr.	+	tr.	+−	+	−	−	−	„ T.N.P.

TABLE X.—*Attempts to Sensitize Animals by Intradermal Injection of Protein Conjugates.*

Date.	Cage No. I.					II.				Date.	III.					IV.				
G.P. No.	1.	2.	3.	4.	.	5.	6.	7.	8.	.	9.	10.	11.	12.	.	13.	14.	15.	16.	.
19.vi.44	175	210	210	210	.	180	180	185	190	. 19.vi .	185	170	155	170	.	200	190	200	170	. Weights (g.).
19.vi.44 to 30.vi.44	6 intradermal doses of antigen				.	Course of sensitization				. 19.vii . to 1.viii	13 intradermal doses of antigen 65				.	13 intradermal doses of antigen 36*b*				. .
12.vii.44 .	+	−	+	+−	.	−	−	−	−	. 9.viii .	++−	+	++	−+	.	+	+−	+	+−	. Serum: precipitins.
	+	−	+	+−	.	++	++	++−	++−	. .:. .	+−	−	−	−	.	tr.	−	−	tr.	. Sensitivity to "tetryl."
	−	+	+	+−	.	+++	++	++−	++−	.	tr.	−	−	−	.	+−	−	−	−	. Sensitivity to T.N.P.
										14.viii .	++	−	++	++	.	−	−	−	−	. Serum: precipitins.
											tr.	tr.	−	+	.	tr.	−	tr.	tr.	. Sensitivity to "tetryl."
											tr.	−	−	+−	.	tr.	−	tr.	−	. Sensitivity to T.N.P.

Doses.—Cage No. I: 0·5 mg./dose of "picryl-guinea-pig-globulin" (untreated). (Antigen 64.)
„ II: (Controls) Standard course of sensitization with "tetryl" in propylene glycol.
„ III: 0·5 mg./dose of "picryl-guinea-pig-globulin" (Antigen 65), treated twice for 2 hours at 37° C. with 5 per cent. glycine at pH 8·5 and dialysed.
„ IV: 0·5 mg./dose of "picryl-horse-serum" (Antigen 36*b*), treated once for 2 hours at 37° C. with 5 per cent. glycine at pH 8·5 and dialysed.

This hypothesis seems the simplest that will explain all the facts. That a particular sort of antigen, a " skin-protein-conjugate," is not essential, is shown by the demonstration, by Landsteiner and Chase (1941), of skin sensitization resulting from intraperitoneal injection of " picryl-stromata," and indeed by all their experiments where the peritoneum and not the skin is the contact tissue ; as also by the experiment detailed here in Table I, of sensitization occurring, almost certainly from absorption *via* the lungs. These experiments also suggest that, just as local production of antigen in the skin is not necessary, neither is local production of antibody.

Light is perhaps thrown on the question by the negative experiment shown in Table X. If the deduction is correct, that picryl-guinea-pig-globulin as such has been ineffective here in producing sensitivity, this experiment at first sight seems to disprove, by direct demonstration, the hypothesis of conjugation between the excitant and tissue protein. It also contrasts with the successful production of sensitivity, by Landsteiner and Chase (1941) by the intraperitoneal injection of full antigen (" picryl-stromata "), treated to ensure the absence of free picryl chloride. These workers do not record any attempt to produce skin sensitivity by *intradermal* injection of picryl-stromata ; in the absence of such an experiment, one is nevertheless forced to the conclusion that the protein " carrier " plays an essential part. The production of skin sensitivity by intraperitoneal injection of picryl stromata, while it is not produced by injection of soluble picrylproteins, either intradermally or intraperitoneally, is evidently due to the special nature of the carrier. Such a particulate antigen, in which the protein, though homologous, is probably somewhat denatured during the preparation, is very different from antigens like those used in the experiment shown in Table X, which were freely soluble and showed no signs of denaturation. This suggests that an antigen acting like a " foreign body " may be an effective sensitizer, where a freely soluble antigen (though still able to produce precipitins) is not ; and one is led back to consider the activities of the wandering cells. In general, indeed, substances such as dead tubercle bacilli, which produce a " foreign body " type of reaction, one, that is, associated with the large mononuclear cells, seem to favour the production of skin-sensitizing antibodies. Possibly the excitant, " tetryl," for example, or picryl chloride, conjugates with and kills the cells in its immediate vicinity (which are then taken up by mononuclears), and these dead cells act as antigens in the same way as the " picryl-stromata " of Landsteiner and Chase ; it may be this reaction, rather than a conjugation of the excitant with proteins dissolved in the body fluids, that is essential for the production of sensitivity.

The hypothesis of a special sort of antibody, which may be carried in cells (though on occasion it may be free in the blood-stream), is unsatisfactory for reasons of economy, but seems to be needed to explain the lack of correlation between circulating antibodies, demonstrable as precipitins and able to produce passive anaphylaxis, and those presumed to be responsible for the skin reaction (but which may also be able to produce passive anaphylaxis when free in the blood-stream). There is a peculiar contrast between the evidently " systemic " nature of the sensitization process, occurring *via* the lymph- and blood-streams, and the localization to tissues of the characteristic " sensitivity " reaction. That this contrast is not merely a concentration effect, depending on a greater delicacy in the skin test, is shown by the absence of skin-reactions in guinea-pigs with plenty of circulating antibodies (Table IV). Some further factor or process must be postulated whereby antibodies are localized in the skin ; and the hypothesis of such antibodies as being produced, and usually fixed, in cells, but in " wandering cells," would help to clear up the difficulties.

SUMMARY.

(1) The problem of sensitivity to "tetryl" (trinitrophenylmethylnitramine) has been investigated. Various methods of sensitization of guinea-pigs are described, and the skin reactions of successfully sensitized pigs to "tetryl" and allied compounds recorded. From these results, it is concluded that tetryl sensitizes by reacting with the recipient's body protein to form an antigenic "picrylprotein."

(2) An experiment is described whereby animals are sensitized by the inhalation of an artificial "smoke" of "tetryl."

(3) Methods of making picrylproteins *in vitro* are given, and by means of these the relation between circulating precipitins and the antibodies responsible for the skin reaction is investigated. It is shown that there is no correlation between these two types of immunological reaction.

(4) Sensitized animals are shown to exhibit anaphylactic phenomena on intravenous injection of picrylproteins, both *in vivo* and *in vitro* by the Dale technique.

(5) The effect of circulating antigen and hapten on skin sensitivity is investigated.

(6) An experiment is described whereby guinea-pigs are shown to be only weakly or not at all sensitized by the injection intradermally of picrylproteins made with either guinea-pig globulin or horse serum.

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